Whole genome sequencing based typing and characterisation of Shiga-toxin producing *Escherichia coli* strains belonging to O157 and O26 serotypes and isolated in dairy farms

Frederique Pasquali,[1] Federica Palma,[1] Marcello Trevisani,[2] Antonio Parisi,[3] Alex Lucchi,[1] Alessandra De Cesare,[1] Gerardo Manfreda[1]

[1]Department of Agricultural and Food Sciences, Alma Mater Studiorum – University of Bologna; [2]Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna; [3]Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Bari, Italy

Abstract

In the present study, the genetic relationships as well as the virulome and resistome of newly sequenced O26 and O157 Shiga-toxin producing *E. coli* (STEC) isolates, collected from dairy farms in Italy, were investigated in comparison to publicly available genomes collected worldwide. The whole genome of Italian isolates was sequenced on Illumina MiSeq Platform. Reads quality control, *de novo* draft genome assembly, species confirmation and the 7-loci Multi-Locus Sequence Type assignment were performed using INNUca pipeline. Reference-based SNPs calling was performed on O157 and O26 genomes, separately, mapping contigs to high-quality finished genomes. Virulence and antimicrobial resistance determinants were detected *in silico* using the tool ABRicate. Phylogenetic reconstructions revealed that genomes clustered mainly based on their 7-loci MLST type. The virulome of tested genomes included 190 determinants. O157 genomes carried *chu* genes associated to heme mediated iron uptake, whereas O26 genomes harboured genes *ybt* associated to siderophore mediated iron uptake. Resistome analysis showed the presence of *tet*(34) on all but one O157 genomes and on only one O26 genomes. Only 4 genomes carried genes associated to multiresistance. In the present study, the genes *chu* and *ybt* were identified as potential biomarker for the differentiation of O157 and O26 serotypes.

Introduction

Shiga-toxin producing *Escherichia coli* (STEC) is an important zoonotic pathogen associated with infections in humans, sometimes with severe symptoms such as haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Griffin and Karmali, 2017). Cattle are considered to be one of the main reservoirs of the bacterium along with sheep and goats (EFSA and ECDC, 2017). In recent years the percentage of confirmed human cases showed a slight increase from 5,680 in 2012 to 6,378 in 2016, confirming STEC infections as the fourth most relevant zoonosis in Europe. The most identified serogroups in humans, food and animals are O157 and O26, with a recent increase in O26 detection (EFSA and ECDC, 2017). STEC O157 and O26 are among the 6 serotypes which have been regulated. After the large O104:H4 outbreak occurred in 2011, a microbiological criterion of "absence in 25 g" of STEC O157, O26, O111, O103, O145 and O104:H4 in sprouted seeds was added to Regulation (EC) No 2073/2005 (Regulation (EC) No 209/2013).

Both STEC O157 and O26 were described as harbouring different essential virulence factors: i) the Shiga-toxin genes *stx1* and *stx2*; ii) the *eae* gene coding for intimin. The genes *stx1* and *stx2* are characterised by three (stx_{1a}, stx_{1c} and stx_{1d}) and seven ($stx_{2a\text{-}g}$) variants respectively, all linked to a different virulence potential with $stx_{2a\text{-}d}$ as strongly associated to sever diarrhoea and HUS (Amézquita-López *et al*., 2017). The gene *eae* is included in the locus of enterocyte effacement (LEE) and described as essential for the attachment of *E. coli* to intestinal epithelial cells (Amézquita-López *et al*., 2017). After the STEC German outbreak of 2011 associated to an *eae*-negative O104:H4 strain, it was observed that other genes might also be effectively involved in the adhesion of *E. coli* to epithelial cells: the plasmid located *aggR* gene or the chromosomally encoded *aaiC* gene. Based on these observations, the combination of *stx2* and *eae* or *stx2* and *aggR/aaiC* was established as reliable predictors of high risk of severe illness (JEMRA, 2016). Virulence genes such as *ehxA* and *hlyA*, coding for haemolysin, were additionally described. In particular *ehxA* was categorised in 4 subtypes with subtype B and C significantly associated to O157 and O26 respectively (Lorenz *et al*., 2013).

Nevertheless, these combinations of genes would have failed to predict the severe illness caused by the "French clone" described as Enterohemorrhagic *E. coli* (EHEC) strain and responsible for sporadic cases from 2010 to 2011. This clone was exclusively *stx2* positive (Delannoy *et al*., 2015; Bielaszewska *et al*., 2013). Moreover, different enterohaemorrhagic clones harbouring none of the *stx* genes were detected and named EHEC-like. Additional combinations of virulence determinants were suggested as potential biomarker predictors of severe illness of EHEC and EHEC-like O26: the *espK* gene with either *espV*, *ureD* and/or *Z2098* and $CRISPR_{O26:H11}$ (Bugarel *et al*., 2011; Delannoy *et al*., 2013; Douëllou *et al*., 2017). In particular, the *esp* genes are linked to type III secreted effector proteins of EHEC, whereas the *ureD* gene is essential for the synthesis of urease accessory protein D linked to the enhancement of acid tolerance during passage though the stomach (Steyert *et al*., 2011). As far as antimicrobial resistance (AMR) is concerned, the prevalence of AMR in STEC differs significantly among Europe. French and English studies reported an AMR prevalence below 20% with the exception of O26 English isolates showing a higher percentage of around

Correspondence: Frédérique Pasquali, Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna, via del Florio 2, 40064 Ozzano dell'Emilia (BO), Italy.
Tel: +39.051.2097862 - Fax: +39.051.2097852.
E-mail: frederique.pasquali@unibo.it

Key words: Shiga-toxin producing *Escherichia coli*, dairy farm, Whole Genome Sequencing, typing, virulome, resistome.

Contributions: AL, AD and MT collected data and performed the lab experiment steps including culture detection, PCR screening test and DNA extraction, AP performed the whole genome sequencing, FrP, FeP and GM designed the study; FeP run the bioinformatics analyses; FrP wrote the manuscript; GM and FeP reviewed the manuscript and contribute to references search.

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36% (Day *et al*., 2017; Um *et al*., 2018). On the other hand, a Spanish study observed 75.3% of isolates to be carrying plasmid-mediated colistin resistance (García *et al*., 2018). A Romanian study on AMR prevalence in young livestock animals observed an increase of multidrug resistance (MDR) from 11% during the 1980s to 40% between 2000 and 2016 (Chirila *et al*., 2017).

Whole Genome Sequencing (WGS) based analyses have recently revealed their great resolution in pathogen typing as well as identification of novel or known genes related to specific phenotypes such as virulence and antimicrobial resistance (Nadon *et al*., 2017; Revez *et al*., 2017; Leopold *et al*., 20014; Oniciuic *et al*., 2018). Studies on whole genome sequencing data aimed to characterise the virulence profiles of O26 or O157 clones are emerging (Holmes *et al*., 2018; Worley *et al*., 2017; Usein *et al*., 2017; Gonzalez-Escalona *et al*., 2016). However, to the best of our knowledge, none has compared the two serotypes.

In the present study, the genetic relationships as well as the virulome and resistome of newly sequenced isolates of O26 and O157 STEC were compared to publicly available genomes.

Table 1. Newly sequenced (labelled EC) and publicly available genomes of O26 and O157 with *stx, eae, hlyA* and *espK* genes related virulence profiles.

Genome	serotype	source	Country	year	stx_{1A}	stx_{1B}	stx_{2A}	stx_{2B}	*eae*	*hlyA*	*espK*
EC1	O26	bulk milk	Italy	2011	-	+	-	-	+	-	+
EC17	O26	bulk milk	Italy	2011	-	-	-	-	+	-	-
EC22	O26	bulk milk	Italy	2011	-	-	-	-	+	+	-
EC3	O26	bulk milk	Italy	2009	-	-	-	-	+	+	-
EC4	O26	milk filters	Italy	2009	-	-	-	-	+	+	-
INNUENDO_STEC_AU_063	O26	human	Austria	2015	-	-	+	+	+	+	+
INNUENDO_STEC_AU_064	O26	human	Austria	2013	-	-	+	+	+	+	+
INNUENDO_STEC_AU_065	O26	human	Austria	2013	-	-	+	+	+	+	+
INNUENDO_STEC_AU_066	O26	human	Austria	2013	-	-	+	+	+	+	+
INNUENDO_STEC_FI_114	O26	human	Finland	2014	-	-	+	+	+	+	-
SAMD00064361	O26	human	Japan	2013	-	+	+	+	+	+	+
SAMN08724660	O26	cattle	US	2009	-	+	+	+	+	+	+
EC12	O157	milk filters	Italy	2009	-	-	-	-	+	+	+
EC2	O157	milk filters	Italy	2011	-	+	+	+	+	-	+
EC33	O157	cattle hide	Italy	2007	-	-	+	+	+	+	-
EC9	O157	milk filters	Italy	2007	-	+	-	-	+	+	-
SAMN01911278	O157	human	Japan	1996	+	+	+	+	+	+	+
SAMN06349171	O157	cattle	Canada	2002	-	+	+	+	+	+	+
SAMN06349172	O157	cattle	Canada	2002	-	+	+	+	+	+	+
SAMN06349173	O157	cattle	Canada	2002	-	+	+	+	+	+	+
SAMN07224767	O157	cattle	Francia	2015	-	+	+	+	+	+	+
INNUENDO_STEC_FI_003	O157	cattle	Finland	2014	-	+	+	+	+	+	+
INNUENDO_STEC_FI_007	O157	cattle	Finland	2012	-	+	+	+	+	+	+
INNUENDO_STEC_FI_015	O157	cattle	Finland	2012	-	+	+	+	+	+	+
INNUENDO_STEC_FI_020	O157	cattle	Finland	2013	-	+	+	+	+	+	+
INNUENDO_STEC_FI_033	O157	environment	Finland	2013	-	+	+	+	+	+	+
INNUENDO_STEC_FI_042	O157	environment	Finland	2014	-	+	+	+	+	+	+
INNUENDO_STEC_FI_067	O157	human	Finland	2014	-	+	+	+	+	+	+
INNUENDO_STEC_FI_071	O157	human	Finland	2012	-	+	+	+	+	+	+
INNUENDO_STEC_FI_077	O157	human	Finland	2014	-	+	+	+	+	+	+
INNUENDO_STEC_FI_084	O157	human	Finland	2010	-	+	+	+	+	+	+
INNUENDO_STEC_FI_088	O157	human	Finland	2013	-	+	+	+	+	+	+
INNUENDO_STEC_FI_092	O157	human	Finland	2010	-	+	+	+	+	+	+
INNUENDO_STEC_FI_094	O157	human	Finland	2009	-	-	+	+	+	+	+
INNUENDO_STEC_FI_102	O157	human	Finland	2011	-	-	+	+	+	+	+
INNUENDO_STEC_FI_106	O157	human	Finland	2013	-	-	+	+	+	+	+
INNUENDO_STEC_FI_109	O157	human	Finland	2013	-	-	+	+	+	+	+
INNUENDO_STEC_FI_111	O157	human	Finland	2013	-	-	+	+	+	+	+
INNUENDO_STEC_FI_116	O157	human	Finland	2014	-	-	+	+	+	+	+
SAMN06159501	O157	human	US	2016	-	-	-	-	-	-	-

Materials and Methods

In the present study, 9 *E. coli* isolates were included. The isolates belong to O157 and O26 serotypes and were collected from bulk milk (n=4), milk filters (n=4) and cattle hide (N=1) (Table 1), between 2007 and 2011 and whole genome sequenced. Part of these isolates was included in a previous study on the detection of STEC in bovine dairy herds in Northern Italy (Trevisani *et al*., 2014). As previously assessed by PCR-based methods, selected isolates carried one or more of three genes: the stx_1 and/or stx_2 genes and/or the *eae* gene (Trevisani *et al*., 2014). For a wider comparison between the two serotypes, 31 publicly available draft (n=29) and complete (n=2) high-quality genomes, belonging to O157 (n=24) and O26 (n=7) STEC serotypes and collected worldwide from humans and cattle sources, were included in the study along with the newly sequenced ones (Table 1). Publicly available genomes were retrieved from NCBI as well as the INNUENDO Sequence Dataset (https://zenodo.org/record/1323690#.W73LV4huYdW) (BioProject n° PRJEB27020). Whole-genomic DNA of Italian isolates was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). The purified DNA concentration and the quality parameter ratio 260/280 were measured by BioSpectrometer fluorescence (Eppendorf). Libraries were built using the TruSeq DNA sample Prep Kit (Illumina, Milan, Italy) and the whole genome of selected isolates was paired-end sequenced using the MiSeq platform (Illumina). Reads of 250 bp on average, were quality checked and *de novo* assembled using the INNUca v1.2 pipeline, which includes SPAdes v3.9 (https://github.com/B-UMMI/INNUca). The pipeline also includes a tool for the *in-silico* characterisation of the 7-loci Multilocus Sequence Type. Reads were submitted to Enterobase (http://enterobase.warwick.ac.uk) under accession numbers: ESC_FA4394AA (EC1); ESC_FA4390AA (EC2); ESC_FA4384AA (EC3); ESC_FA4387AA (EC4); ESC_FA4385AA (EC9); ESC_FA4395AA (EC12); ESC_FA4389AA (EC17); ESC_FA4391AA (EC22); ESC_JA4691AA (EC33).

SNP calling was performed on O157 and O26 draft genomes, separately using the open source snippy v3.2 pipeline with default settings (https://github.com/tseemann/snippy). High-quality complete genomes *E. coli* O157:H7 str. Sakai (EHEC) (Ref Seq NC_002695) and *E. coli* O26:H11 str. 11368 (Ref Seq NC_013361.1) were used as references for SNP calling of O157 and O26 genomes respectively. For each serotype, an alignment of core genome SNPs was generated by snippy and used to infer a Maximum Likelihood (ML)-based high-resolution phylogeny using the iQTree software (Nguyen *et al*., 2015). In order to evaluate the genetic distance among different STEC ST, phylogenetic trees were graphically represented with iTOL viewer (https://itol.embl.de/). The most genetically distant genome for each serotype, counting several tens of thousands of SNPs, was used to root the ML-trees.

Analyses of virulome and resistome of all genomes were performed using ABRicate (https://github.com/ tseemann/abricate/). With this tool, a BLAST search of genes included in the Virulence Factors Database (VFDB) and the Resfinder database was performed on *de novo* assemblies of newly sequenced as well as publicly available selected genomes (http://www.mgc.ac.cn/VFs/main.htm; https://cge.cbs.dtu.dk/services/ResFinder/). In particular, the VFDB database includes 2,606 curated genes related to virulence factors whereas the Resfinder database includes 1,723 genes related to antimicrobial resistance (Chen *et al*., 2016; Zankari *et al*. 2013).

Results and Discussion

The draft genome sequences of 9 newly sequenced STEC isolates, collected from bovine dairy farms in Italy over four years (2007-2011), passed the QA/QC measures defined by INNUca pipeline. Draft genomes included from 93 to 345 contigs with a final coverage between 42X and 79X and N50 values ranging from 76377 to 208613 (Table S1).

In order to evaluate the genetic relationships among newly sequenced Italian genomes of cattle origin in comparison to public genomes isolated from cattle as well as humans worldwide, SNPs-based phylogenetic analyses were performed using snippy on the whole genome of the O157 and O26 *E. coli* strains separately. The resulting ML-trees show genomes essentially clustered based on their ST with ST11 as the most prevalent O157 ST and ST21 and ST29 as the most prevalent O26 STs (Figure 1). These STs have been already described as associated to EHEC strains (Bielaszewska *et al*., 2013; Wang *et al*., 2014).

Overall, the SNPs differences encountered within ST11 genomes of O157 isolates in relation to the reference were between 854 and 2,861 (Figure 1A). A smaller genetic distance was observed among O26 genomes of ST21 against the selected reference, with a SNPs count between 257 and 1,615 (Figure 1B), while ST29 genomes cluster showed a SNPs count ranging from 4492 to 5336 (Figure 1B). Overall, within each serotype a high genetic diversity was observed with no specific clustering of genomes based on the year or country of isolation as well as source.

The virulome and resistome of a total of 40 STEC genomes belonging to O26 and O157 serotypes were compared. The virulome of draft genomes included 190 virulence determinant genes. Among the 40 genomes tested, only one O157 isolate was positive for stx_{1a} whereas 22 were positive for stx_{1b}, 32 for stx_{2a} and stx_{2b}, 39 for *eae*, 36 for *hlyA* and 32 for *espK*. Five genomes did not carry stx_1 or stx_2 but carried the *eae* and/or *hlyA* genes with one genome additionally carrying the *espK* gene suggesting these five as potential EHEC-like isolates. One publicly available O157 genome did not carry any of the five virulence genes (Table 1). Similar patterns were observed in cattle and human genomes confirming the cattle genomes as potentially pathogenic for humans as previously suggested (EFSA and ECDC, 2017; Trevisani *et al*., 2014).

The heatmap of all identified 190 virulence genes is reported in Figure 2. Comparing O157 and O26 genomes, all O157 and none O26 carried *chu* genes, homologous to *shu* genes of *Shigella dysentariae*, related to the use of the heme group of haemoglobin as iron source. This mechanism was described as an efficient strategy for iron acquisition during an ongoing infection (Torres and Payne, 1997; Wyckoff *et al*., 1998; Braun, 2001). Both heme and haemoglobin were already described as significantly stimulating the growth of *E. coli* O157 and production of enterohaemolysin in comparison to non-O157 strains (Law *et al*., 1995). This observation suggests that the heme mediated iron uptake is specific for O157 serotype. Moreover, O157 genomes carried a higher number of *esp* genes: *espR*, *espX* e *espY* genes, involved in the secretion system of type III associated to the survival of the pathogen within the host cell (Galán and Wolf-Watz, 2006). On the other hand, O26 carried *flhA*, a gene associated to the biosynthesis of flagella involved in the first steps of adhesion and invasion (Haiko and Westerlund-Wikström, 2013), which was not detected in any O157 genomes. Finally, all O26 and none O157 genomes carried eight *ybt* genes (*ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtS*, *ybtT*, *ybtU* e *ybt*X) associated to the acquisition of iron from yersinabactin, a highly relevant siderophore for the hyper-virulence of *Yersinia enterocolitica* (Pelludat *et al*., 1998). Further stud-

ies should be performed in order to evaluate whether the siderophore mediating iron uptake is significantly linked to the recent emergence of O26 as the serotype most frequently associated to haemolytic uremic syndrome (HUS) in children (EFSA and ECDC, 2017). Finally, all O26 genomes but one carried the *fyuA* gene along with the *irp1* and *irp2* genes. These *irp* genes are included in the gene cluster related to the biosynthesis of yersinabactin while *fyuA* encodes for the outmembrane receptor for this siderophore (Pelludat *et al*., 1998).

Regarding the resistome, a limited number of AMR-associated genes to antimicrobial resistance was detected (Table 2). In particular, among the newly sequenced genomes, two O26 genomes carried the *tet*(C) gene, whereas one O26 and three O157 genomes carried the *tet*(34) gene with a gene coverage of 76,34%. Among the publicly available genomes, *tet*(34) gene was detected on 23 O157 genomes, along with *aph*(6)-Id, *strA*, *sul2* and *tet*(B) in three of them. These three genomes were related to isolates collected from cattle in Canada in 2002. These genes are associated to aminoglycoside, streptomycin, sulphonamides and tetracycline resistance respectively. An additional potentially multiresistant isolate belonging to O26 serotype and collected from humans in Japan, carried the *aph*(3'')-Ib, *bla*TEM-30 and *sul2* conferring resistance to aminoglycosides, beta-lactams and sulphonamides respectively. The low detection rate of AMR genes observed across the 40 screened genomes, regardless of their serotype, was in accordance to previously reported data (Day *et*

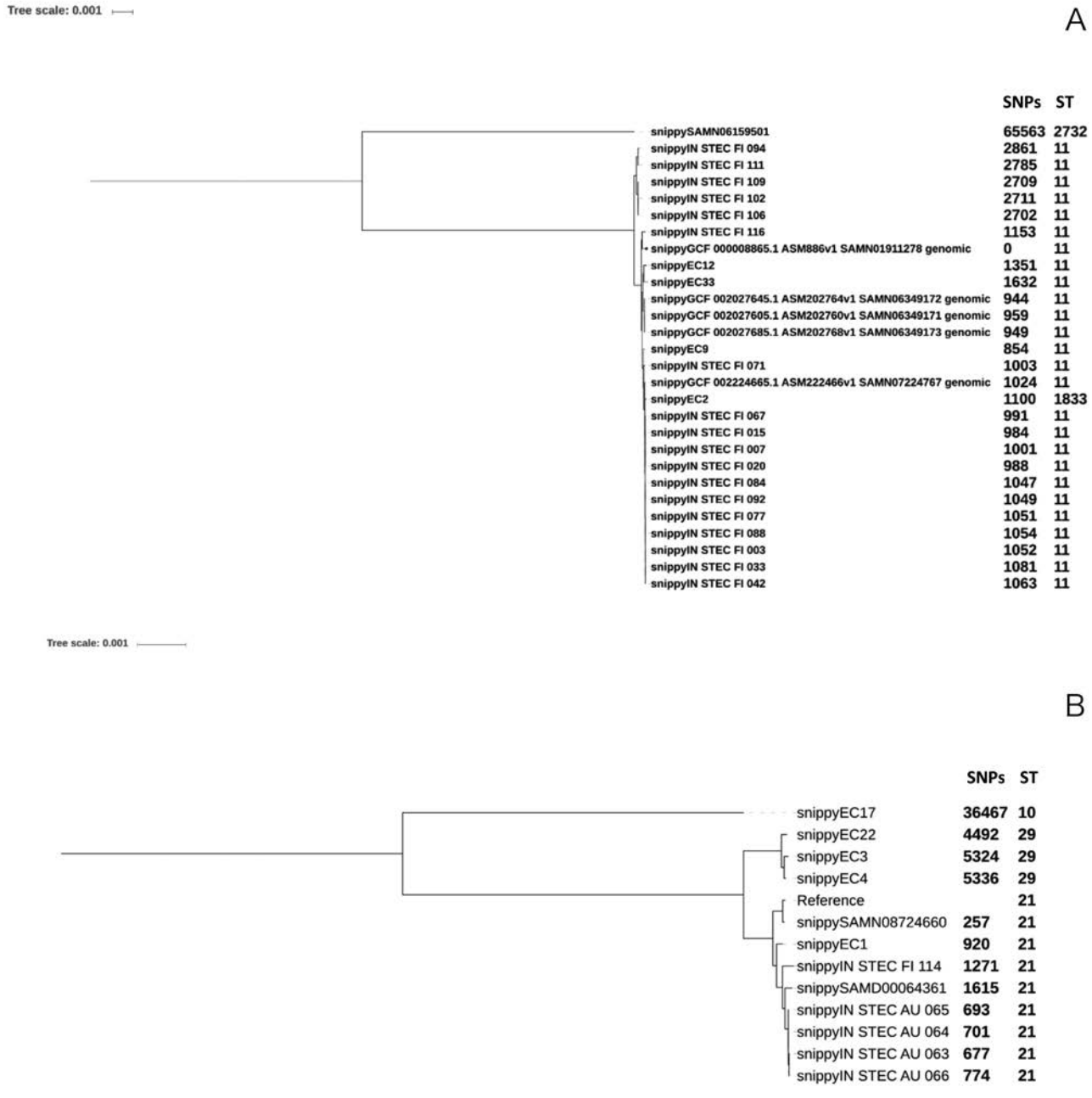

Figure 1. Maximum likelihood phylogeny based on whole genome SNPs of O26 genomes (A) and O157 genomes (B). The number of SNPs differences to the reference is reported in the first column (SNPs) and 7-*loci* MLST type on the second column (ST).

al., 2017).

Overall, all but one O157 and none but one O26 genomes harboured the *tet*(34) gene. No differences were observed both on the virulome and resistome of human *versus* cattle STEC genomes confirming the phylogenetic tree outputs which clustered together genomes from both sources.

Conclusions

In order to compare draft genomes of the two mostly isolated serotypes associated to haemorrhagic colitis and haemolytic uremic syndrome in humans, the genetic relationships as well as the virulome and resistome of 40 STEC genomes were assessed. Newly sequenced as well as publicly available genomes of O26 and O157 serotypes isolated from cattle and humans worldwide were included in the study showing no differences in terms of genetic distance as well as of virulome and resistome compositions of human *versus* cattle genomes. Based on the virulome analysis, the presence of different virulence genes in O26 and O157 associated to siderophore and heme mediated iron uptake systems, respectively, was

Table 2. Antimicrobial resistance determinant genes of newly sequenced (labelled EC) and publicly available genomes of O26 and O157.

Genome	*aph(3'')-Ib*	*aph(6)-Id*	*blaTEM-1C*	*blaTEM-30*	*strA*	*sul2*	*tet(34)**	*tet(B)*	*tet(C)*
EC1	-	-	-	-	-	-	-	-	-
EC17	-	-	-	-	-	-	+	-	-
EC22	-	-	-	-	-	-	-	-	-
EC3	-	-	-	-	-	-	-	-	+
EC4	-	-	-	-	-	-	-	-	+
INNUENDO_STEC_AU_063	-	-	-	-	-	-	-	-	-
INNUENDO_STEC_AU_064	-	-	-	-	-	-	-	-	-
INNUENDO_STEC_AU_065	-	-	-	-	-	-	-	-	-
INNUENDO_STEC_AU_066	-	-	-	-	-	-	-	-	-
INNUENDO_STEC_FI_114	-	+	-	-	+	-	-	-	-
SAMD00064361	+		+	-	-	+	-	-	-
SAMN08724660	-	-	-	-	-	-	-	-	-
EC12	-	-	-	-	-	-	+	-	-
EC2	-	-	-	-	-	-	+	-	-
EC33	-	-	-	-	-	-	+	-	-
EC9	-	-	-	-	-	-	+	-	-
SAMN01911278	-	-	-	-	-	-	+	-	-
SAMN06349171	-	+	-	-	+	+	+	+	-
SAMN06349172	-	+	-	-	+	+	+	+	-
SAMN06349173	-	+	-	-	+	+	+	+	-
SAMN07224767	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_003	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_007	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_015	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_020	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_033	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_042	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_067	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_071	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_077	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_084	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_088	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_092	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_094	-	-	-	+	-	-	+	-	-
INNUENDO_STEC_FI_102	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_106	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_109	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_111	-	-	-	-	-	-	+	-	-
INNUENDO_STEC_FI_116	-	-	-	-	-	-	+	-	-
SAMN06159501	-	-	-	-	-	-	-	-	-

**tet(34)*: detected with 76.34 % of coverage

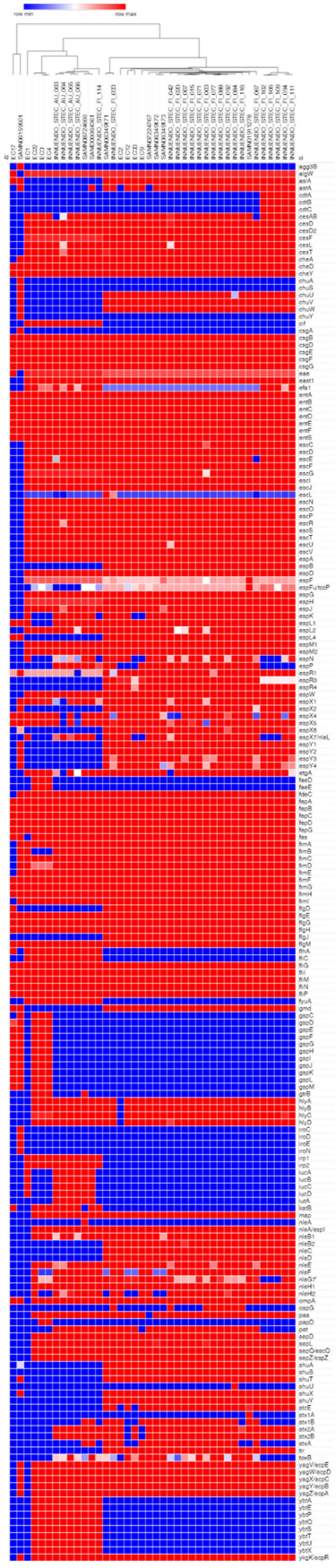

Figure 2. Heatmap of the virulome of O157 and O26 genomes. Colours from blue to red correspond to a range of 0-100% coverage of the gene sequences of the Virulence Finder Database.

observed. These genes, *chu* and *ybt*, could be used as biomarkers for the identification of O157 and O26 genomes. Further analyses are also required to better understand how the iron and other metals (*i.e.* copper) uptake system impact on the pathogenicity of the strain belonging to these two haemorrhagic serotypes. Additional investigations based on genome-wide association study (GWAS) including a higher number of publicly available genomes should be performed in order to confirm the statistically significant relevance of the serotype-specific genes identified in this study.

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