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

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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 (EFSWA 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 (EFSWA 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 stxl and stx2 are characterised by three (stx2a, stx2b, and stx2c) and seven (stx2a-d) variants respectively, all linked to a different virulence potential with stx2a-d as strongly associated to severe diarrhoea and HUS (Amézquita-López et al., 2017). The gene eae is included in the locus of enteroocyte 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 ehaA and hlyA, coding for haemolysin, were additionally described. In particular ehaA 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 enterohemorrhagic 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 (Bugar et al., 2011; Delannoy et al., 2013; Douéllou 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 through 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

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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 (Garcia 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; Oniciuc 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).

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

| Genome             | serotype | source   | Country | year | stx1A | stx1B | stx2A | stx2B | eae   | hlyA | espK |
|--------------------|----------|----------|---------|------|-------|-------|-------|-------|-------|------|------|
| INNUENDO_STEC_FI_063 | O26      | human    | Austria | 2015 | -     | +     | +     | +     | +     |
| INNUENDO_STEC_FI_064 | O26      | human    | Austria | 2013 | -     | +     | +     | +     | +     |
| INNUENDO_STEC_FI_065 | O26      | human    | Austria | 2013 | -     | +     | +     | +     | +     |
| INNUENDO_STEC_FI_066 | O26      | human    | Finland | 2014 | -     | +     | +     | +     | +     |
| EC1                | O157     | milk filters | Italy   | 2011 | -     | -     | +     | +     | +     |
| EC2                | O157     | milk filters | Italy   | 2011 | -     | +     | +     | +     | +     |
| EC3                | O157     | milk filters | Italy   | 2007 | -     | +     | +     | +     | +     |
| SAMN06349171       | O157     | cattle    | Canada  | 2002 | -     | +     | +     | +     | +     |
| SAMN06349172       | O157     | cattle    | Canada  | 2002 | -     | +     | +     | +     | +     |
| SAMN0662724767     | 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 | 2013 | -     | +     | +     | +     | +     |
| 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 | -     | +     | +     | +     | +     |
| SAMN06619501       | 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 stx1 and/or stx2 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/repository/1323690#.W73V4huYdW) (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 (Nuygen 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 7637 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 with a final coverage between 42X and 79X and N50 values ranging from 7637 to 208613 (Table S1).

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 dysenteriae, 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 espT 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 flaA, 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, ybtB, ybtC, ybtD, ybtF, ybtG, ybtI, ybtU) associated to the acquisition of iron from yersiniabactin, a highly relevant siderophore for the hyper-virulence of Tersinia 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*, *blaTEM-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 al., 2009).

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 uraemic 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 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               | +          | +         | -         | -         | -    | -    | -        | -      | -      |
| SAMN0824660                | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| EC12                        | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| EC2                         | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| EC33                        | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| EC9                         | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| SAMN01912178               | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| SAMN06349171               | -          | +         | -         | -         | +    | +    | +        | +      | -      |
| SAMN06349172               | -          | +         | -         | -         | +    | +    | +        | +      | -      |
| SAMN06349173               | -          | +         | -         | -         | +    | +    | +        | +      | -      |
| SAMN0724767                | -          | -         | -         | -         | -    | +    | -        | -      | -      |
| 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        | -          | -         | -         | -         | -    | -    | -        | -      | -      |
| SAMN0619501                | -          | -         | -         | -         | -    | -    | -        | -      | -      |

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