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Genetic diversity and pathogenic potential of Shiga toxin-producing *Escherichia coli* (STEC) derived from German flour

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) can cause severe human illness, which are frequently linked to the consumption of contaminated beef or dairy products. However, recent outbreaks associated with contaminated flour and undercooked dough in the United States and Canada, highlight the potential of plant based food as transmission routes for STEC. In Germany STEC has been isolated from flour, but no cases of illness have been linked to flour.

In this study, we characterized 123 STEC strains isolated from flour and flour products collected between 2015 and 2019 across Germany. In addition to determination of serotype and Shiga toxin subtype, whole genome sequencing (WGS) was used for isolates collected in 2018 to determine phylogenetic relationships, sequence type (ST), and virulence-associated genes (VAGs).

We found a high diversity of serotypes including those frequently associated with human illness and outbreaks, such as O157:H7 (*stx2c/d, eae*), O145:H28 (*stx2a, eae*), O146:H28 (*stx2b*), and O103:H2 (*stx1a, eae*). Serotypes O187:H28 (ST200, *stx2g*) and O154:H31 (ST1892, *stx1d*) were most prevalent, but are rarely linked to human cases. However, WGS analysis revealed that these strains, as well as, O156:H25 (ST300, *stx1a*) harbour high numbers of VAGs, including *eae, nleB* and *est1a/sta1*.

Although STEC-contaminated flour products have yet not been epidemiologically linked to human clinical cases in Germany, this study revealed that flour can serve as a vector for STEC strains with a high pathogenic potential. Further investigation is needed to determine the sources of STEC contamination in flour and flour products particularly in regards to these rare serotypes.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne pathogens (Caprioli et al., 2014), that can cause severe human diseases like diarrhoea, haemorrhagic colitis and the haemolytic-uraemic syndrome (HUS) (Scheutz, 2014). Early investigations on human clinical cases mainly focused on serotype O157:H7, but a variety of non-O157 serogroups like O26, O45, O91, O103, O111, O121, O145 and O146 have also been isolated regularly from human clinical samples (Mathusa et al., 2010; Smith et al., 2014). However, STEC exhibit diversity of genome content, with a complex population structure resulting from horizontal gene transfer (Denamur et al., 2021). Individual strains of the same serotype apparently vary in their potential to cause severe illness, and the virulence-associated genes (VAGs) they carry. The pathogenic potential of STEC strains has been correlated with the Shiga toxin subtype, as well as, the (co-)occurrence of other VAGs (Bugarel et al., 2011; Fuller et al., 2011; Mathusa et al., 2010; Santos et al., 2020; Soderlund et al., 2016). Furthermore, so-called hybrid strains displaying virulence characteristics of STEC together with those of other *E. coli* pathotypes, such as enteropathogenic *E. coli* (EPEC), or

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enterotoxigenic *E. coli* (ETEC), are not as rare as previously thought (Bai et al., 2019; Bibbal et al., 2014; Blanco et al., 2005; Feng et al., 2010; Granobles Velandia et al., 2012; Nyholm et al., 2015; Wieler et al., 1996).

Cattle and small ruminants are important hosts for STEC (Brashears and Chaves, 2017; Bruyand et al., 2019; Gyles, 2007). As such, many human outbreaks are linked to the consumption of contaminated beef and dairy products (Farrokh et al., 2013; Hussein and Bollinger, 2005; Hussein and Sakuma, 2005). However, STEC are also increasingly reported in non-animal food products such as fresh produce and flour (Crowe et al., 2017; Feng, 2014; Gill et al., 2019; Olaimat and Holley, 2012). In 2011 a large outbreak occurring in Germany and France was linked to sprouts as the most likely source of infection (Buchholz et al., 2011; Soon et al., 2013). Furthermore, between 2009 and 2017 flour, raw or undercooked dough and baking products were identified as STEC transmission vehicles for several human cases in the United States and Canada (Crowe et al., 2017; Gieraltowski et al., 2017; Gill et al., 2019; Morton et al., 2017; Neil et al., 2012).

In Germany, STEC outbreaks linked to flour and flour products have not been reported. Nevertheless, 39% of flour samples collected in a study in Germany between 2014 and 2017 were found *stx*-gene positive by real-time PCR (Mäde et al., 2017), but the genetic diversity of these STEC strains has not been determined.

In this study, we analyzed STEC isolated from flour, ready-mixes and flour products thereof isolated between 2015 and 2019 by official laboratories in the framework of food surveillance programs in Germany. These strains were collected to the strain collection of the National Reference Laboratory for *Escherichia coli* (NRL-*E. coli*) at the German Federal Institute for Risk Assessment (BfR) and the Robert Koch Institute (NRC-RKI). The aim of this study was to investigate the genetic diversity of these flour-derived STEC strains using serological, molecular, and whole genome sequencing (WGS) analysis and to assess their pathogenic potential.

2. Materials and methods

2.1. Strains

Shiga toxin-producing *Escherichia coli* (STEC, $n = 123$, without duplicate samples) were isolated from flour, ready-mixes and flour products thereof, collected during official food surveillance between 2015 and 2019 across Germany (supplementary table). STEC were isolated by official laboratories and sent to the NRL-*E. coli* or the NRC-RKI for confirmation and further characterization. In 2018, a Federal Surveillance Program (FSP) coordinated by the Federal Office of Consumer Protection and Food Safety (BVL) targeted the voluntary microbiological analyses of various flour types and flour products derived from mills, food retailers and bakeries. Isolates were derived from ten out of 16 German federal states.

2.2. Laboratory analyses

Samples were streaked on selective agar plates, *i.e.* Endo agar (Oxoid, Germany), Sorbitol MacConkey agar (Oxoid, Germany), Fluorocult® *E. coli* O157:H7 agar (Merck, Germany), CHROMagar™ STEC (Mast Diagnostica GmbH, Germany), and Enterohemolysin agar (SIFIN GmbH, Germany) to test for mixed cultures. Serotypes and virulence genes for the obtained isolates were determined for single colonies as part of our routine testing (Beutin et al., 2007a; Prager et al., 2011). Serotyping of the O antigen was carried out according to Ørskov and Ørskov (Ørskov and Ørskov, 1984), the H type was identified by serotyping or by using conventional PCR of the flagellin gene *fliC* and subsequent enzymatic digestion of the PCR product as well as Sanger sequencing (Machado et al., 2000). Shiga toxin genes *stx1* and *stx2* (including *stx* subtyping), intimin gene *eae* (for enteropathogenic *E. coli*, EPEC), hemolysin A gene (*hlyA*, also known as *ehxA*) and the non-LEE (locus of enterocyte effacement)-encoded effector gene *nleB* were determined by real-time PCR and conventional PCR as described (Beutin et al., 2007b; Gunzer et al., 1992; Leung et al., 2003; Scheutz et al., 2012; Tzschoppe et al., 2012).

2.3. DNA preparation and whole genome sequencing

Total genomic DNA of 56 isolates from 2018 was prepared using the PureLink® Genomic DNA Mini Kit (Invitrogen, USA) according to the manufacturers' instructions. Sequencing libraries were prepared with the Nextera DNA Flex Library Prep kit (Illumina, San Diego, USA) and paired-end sequencing was performed on the Illumina MiSeq benchtop sequencer (Miseq Reagent v3, 600 cycle Kit) or on the Illumina NextSeq 500 benchtop sequencer (NextSeq 500/550 v2.5, 300 cycle Kit). Raw Illumina reads were trimmed and *de novo* assembled using the AQUAMIS pipeline (https://gitlab.com/bfr_bioinformatics/AQUAMIS/), developed in-house, which implements fastp for trimming (Chen et al., 2018) and unicycler (Wick et al., 2017) or shovill (https://github.com/tseemann/shovill, based on Spades) for assembly. The AQUAMIS pipeline performs mash v 2.1 for reference search (Ondov et al., 2016), as well as quast v 4.6.3 for assembly quality control (https://github.com/ablab/quast). Assembled genome data were deposited at the National Center for Biotechnology Information database https://www.ncbi.nlm.nih.gov/ under the BioProject PRJNA715185 (Accession numbers SAMN18335720 to SAMN18335775).

2.4. Phylogenetic analyses and detection of virulence genes

In silico analysis of multi locus sequence types (MLST) (Wirth et al., 2006) and core-genome (cg) MLST based on 2513 loci (https://enterobase.warwick.ac.uk/species/index/ecoli) were carried out using Ridom Seqsphere+ v7.0.4 (Ridom GmbH, Germany). For four strains new MLST STs were assigned by uploading the raw sequencing data to the Enterobase database (https://enterobase.warwick.ac.uk) (Zhou et al., 2020). A minimum spanning tree (MST) was calculated based on the *E. coli* cgMLST v1.0 data using Ridom Seqsphere+ v7.0.4. The MST was distance based on 2513 loci with pairwise ignoring missing values. The cluster alert distance (AD) was set to 10 alleles.

In addition to routine testing of isolates for *stx1, stx2*, *eae*, and *nleB* genes, whole genome sequences were further analyzed for 297 putative virulence genes incoporated in the Ridom Seqsphere+ v7.0.4 software (Ridom GmbH, Germany; required identity to reference sequence: 80%, required percentage aligned to reference sequence: 60%) and the BioNumerics 7.6.3 *E. coli* functional genotyping plug-in version 1.2 (Applied Math, Belgium; Blast minimum sequence identity 90%, Blast minimum length for coverage 60%) based on databases from the Virulence Factor Database (VFDB, http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Escherichia) and the Center for Genomic Epidemiology (http://www.genomicepidemiology.org).

3. Results

3.1. STEC strains

Between 2015 and 2019, 138 isolates from flour, ready-mixes and flour products thereof were sent to the NRL-*E. coli* and NRC-RKI for sero- and molecular typing (supplementary table). Duplicate isolates with the same serotype and virulence genes (*stx1, stx2, eae* and *nleB* determined by routine testing) and derived from the same sample were excluded from further comparisons (except those from 2018 to determine the intra sample diversity by WGS). For seven samples, presumptive duplicate isolates were assigned to different serotypes and/or harboured different *stx* gene variants. In total, 123 isolates were included in the presented study. Numbers of investigated isolates each year are shown in Table 1.

Isolates were sent to the NRL-*E. coli* and NRC-RKI between 2015 and

Table 1
Numbers of isolates investigated in the study.

Year	Isolates provided	Isolates excluded from further analysis[a]	Final number
2015	13	2	11
2016	11	3	8
2017	48	8	40
2018	58	2	56[b]
2019	8	0	8
Total	138	15	123

[a] Duplicate isolates with the same serotype and virulence genes derived from the same sample.
[b] Isolates for whole genome sequencing.

2019 from laboratories in ten different German Federal states. In 2018 the FSP was carried out resulting in a higher number of isolates being sent to the NRL-*E. coli* for further investigation. Numbers and distribution of STEC across the German Federal states are shown in Fig. 1. Of note, the numbers of STEC isolated are not correlated to a contamination rate, as STEC isolates were sent to the NRL-*E. coli* on a voluntarily basis.

STEC strains characterized in this study were isolated from different flour, ready-mixes and flour products, of which wheat flour was most frequent (Table 2), totalling nearly two thirds of the analyzed isolates.

3.2. Serotypes

Analyses of the 123 STEC strains identified the presence of 20 different serogroups, primarily belonging to a single serotype, except O79 and O8, where two different serotypes were identified each (O79:

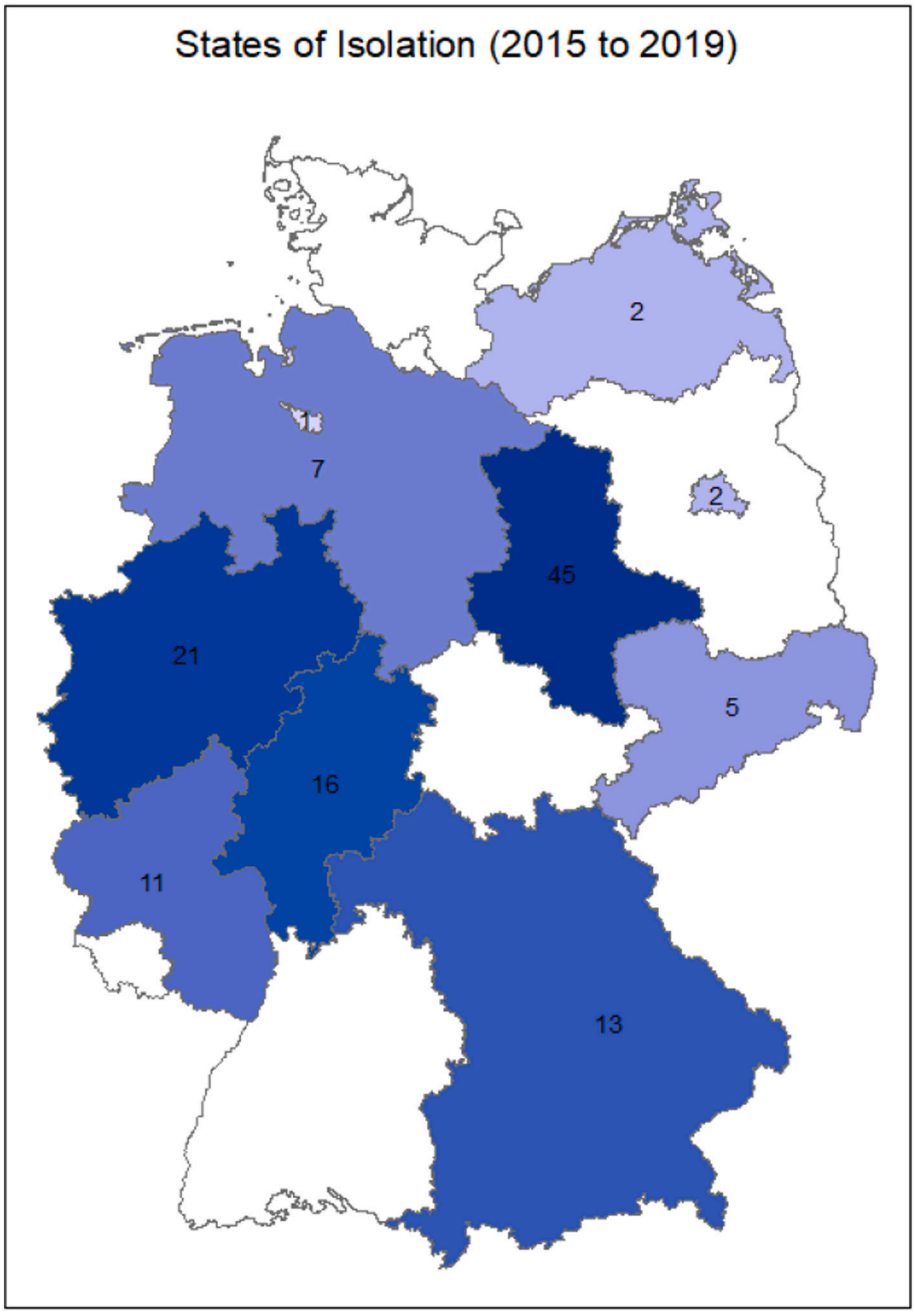


Fig. 1. Numbers of STEC isolates collected by the German Federal states and sent to the NRL-*E. coli* and NRC-RKI between 2015 and 2019. States displayed in white did not sent isolates to the NRL-*E. coli* or NRC-RKI.

Table 2
Numbers of isolates investigated in the study and distribution across the different types of flour and flour derived products.

Matrix	Numbers of isolates (%)
Wheat flour	75 (60.9)
Rye flour	30 (24.4)
Spelt flour	5 (4.6)
Spelt shortcrust	1 (0.8)
Ready-mixes	5 (4.6)
Other flour products[a]	7 (5.7)

[a] Grain or flour product not further specified.

H14, O79:H23, O8:H9, O8:H19). Of the 27 different serotypes (including O not typeable/rough) O187:H28, O154:H31, O11:H48, and O36:H14 were predominant (Fig. 2, supplementary table). Interestingly, strains of these four serotypes were isolated from at least three different flour matrices. In addition, STEC of serotypes O157:H7, O145:H28, O146:H28, and O103:H2 were isolated.

3.3. Shiga toxin subtyping and virulence genes

All 123 isolates carried either Stx1- or Stx2-encoding genes but none were positive for both Shiga toxin variants. Shiga toxin gene variants were detected as follows: *stx1a* ($n = 8$), *stx1c* ($n = 1$), *stx1d* ($n = 44$), *stx2a* (n = 1), *stx2b* ($n = 17$), *stx2c/d* ($n = 2$, the typing method used was not able to distinguish between *stx2c* and *stx2d*), *stx2e* ($n = 3$) and *stx2g* ($n = 47$).

Ten STEC stains were found to carry both *eae* and *nleB* genes. Interestingly, seven out of these ten isolates carried *stx1a* and belonged to serotypes O156:H25 ($n = 5$) and O103:H2 (n = 2). The remaining three isolates were of serotype O157:H7 (n = 2) and O145:H28 ($n = 1$) harbouring *stx2c/d* and *stx2a*, respectively.

WGS was applied to the 56 STECs isolated in 2018 and the sequencing data was screened for 297 putative VAGs as incorporated in the Ridom and BioNumerics software (supplementary table). The number of VAGs detected in individual strains varied between 35 and 114. The isolated O157:H7 strain from 2018 was found to carry the highest number of putative VAGs ($n = 114$), followed by O156:H25 strains ($n = 87, 84, 82$), and the O103:H2 strain ($n = 83$). In addition, 21 isolates (37.5%) were positive for the gene encoding the heat stable toxin (*estIa/sta1)* commonly found in ETEC (Blanco et al., 1991; Dubreuil et al., 2016). Twenty-five strains (44.6%) were found to carry the enteroaggregative *E. coli* heat stable enterotoxin-1 gene (*astA*) and 15 isolates (26.8%) were positive for both *estIa/sta1* and *astA*. In 28 isolates (50%) the enterohemolysin gene (*ehxA/hlyA*) was detected and 20 isolates (35.7%) were positive for F17 fimbriae. Afimbrial adhesins-encoding genes (*afaA/B/F*) were detected in the four O11:H48 isolates and the gene encoding the enterohemorrhagic *E. coli* factor for adherence (*efa1*) in the O103:H2 isolate from 2018. The catalase-peroxidase gene *katP* was found in the O157:H7 and three O187:H28 isolates. The genes *escC-V* (type III secretion system), *esp/ces* (type III secretion system), *etgA* (muraminidase), *etpD* (type III secretion system), *paa* (porcine attaching and effacing-associated gene), *sep* (type III secretion system) and *tir* (translocated intimin receptor), as well as, *eae* and *nleB* genes were detected in six STEC strains belonging to serotypes O157:H7 (n = 1), O103:H2 (n = 1) and O156:H25 ($n = 4$). The *iha* gene (adhesion) was found in the five O146:H28 isolates, as well as, the O157:H7 isolate. The *toxB* gene was exclusively detected in the O157:H7 isolate. Based on combined BLAST analyses of 15 contigs (88,250 bp) of the O157:H7 strain *toxB, katP, ehxA* and *espP* appear to be located on a single plasmid showing a 99% coverage and 99.99% sequence identity to plasmid pO157 (Acc.no. CP040317.1).

3.4. Phylogenetic analysis

Using WGS the 56 isolates were assigned to 18 different MLST (Warwick scheme) of which ST200 (Cplx40) was most prominent ($n = 13$). These isolates belonged to serotypes O187:H28 (n = 11) or O175:H28 ($n = 2$) all of which harboured *stx2g*. Strains of the same serotype were found in most cases to share a common MLST (Fig. 3). Interestingly, four STEC were assigned a new MLST, ST10828 (serotype O11:H48) not previously described.

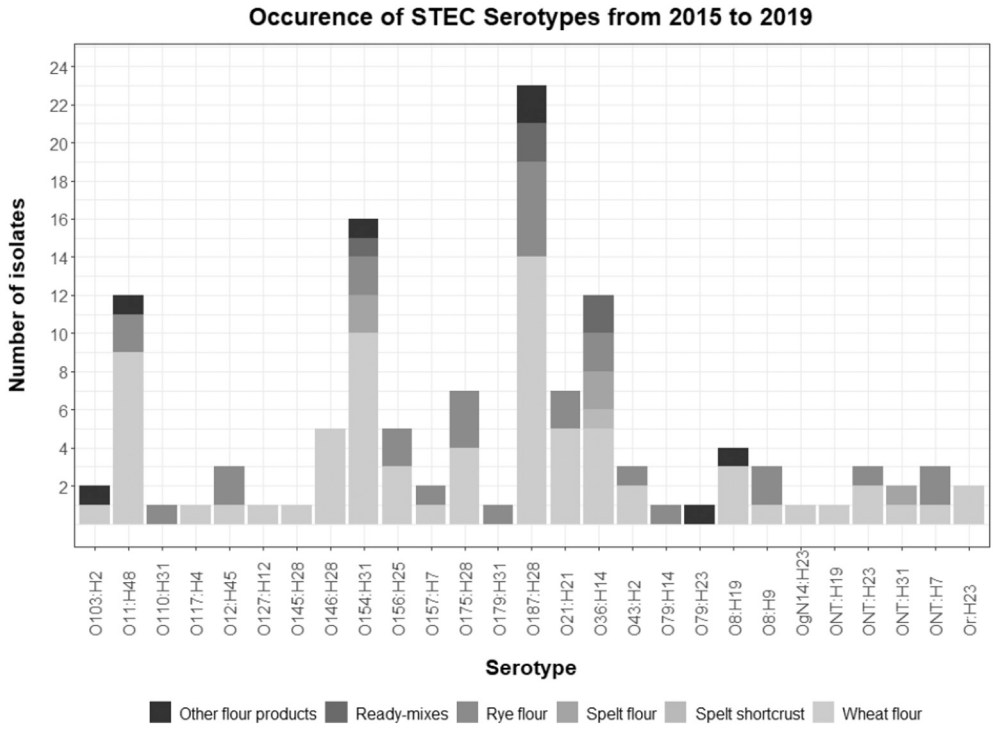
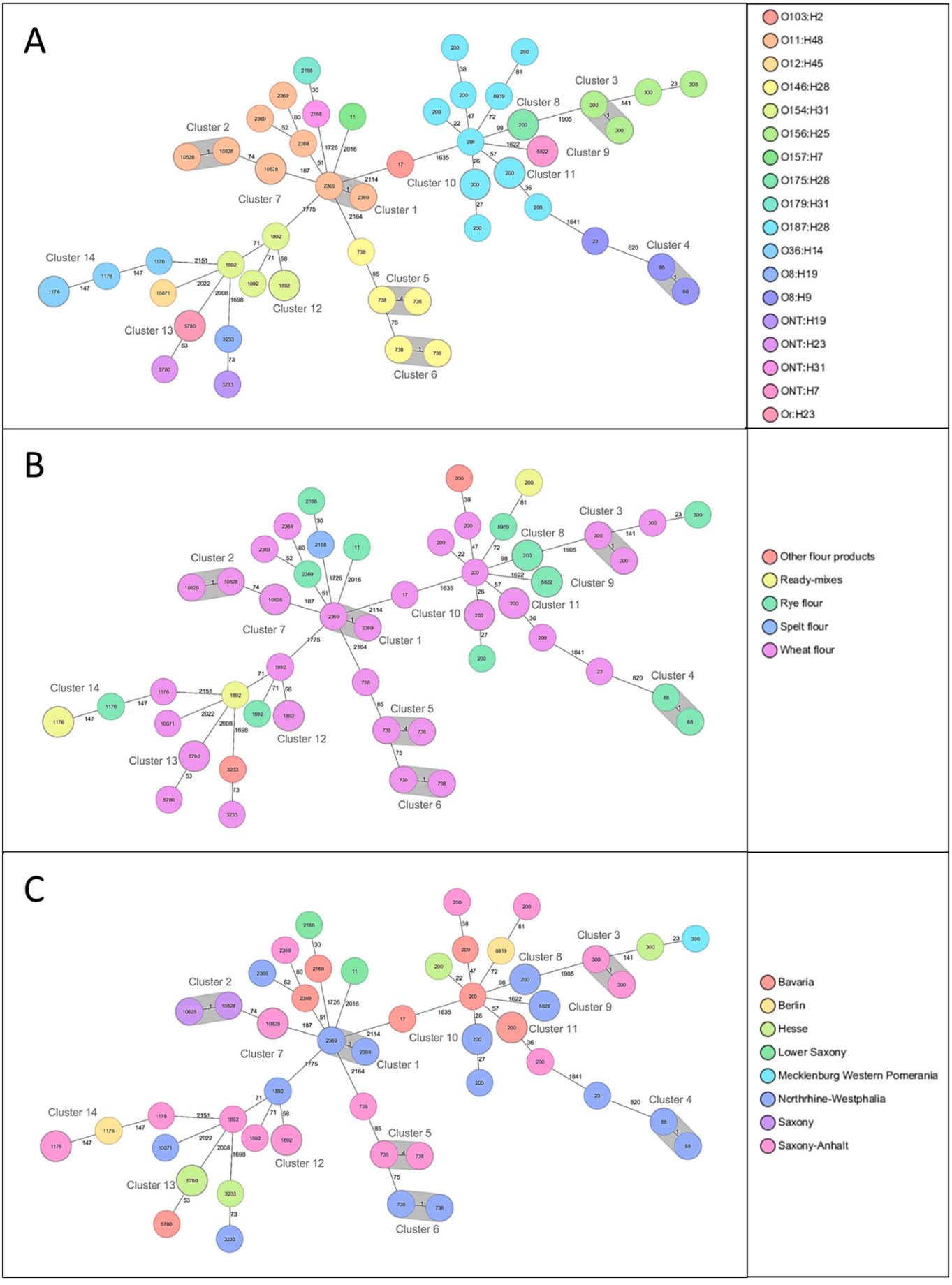


Fig. 2. Numbers of STEC serotypes identified in flour/flour products in Germany (2015–2019); OgN: novel O genotype, ONT: O-antigen not typeable, Or: O-antigen rough.



(caption on next page)

Fig. 3. a-c. Minimum spanning tree (MST) for 56 STEC isolates from 2018 based on the *E. coli* cgMLST v1.0 (2513 columns, pairwise ignoring missing values, logarithmic scale, Cluster-Alert distance: 10 alleles; highlighting in colours the distributions of serotypes (A), the source of isolation (B) and states of isolation (C). The STs of the *E. coli* MLST Warwick v1.0 scheme were used for node description. Clusters highlighted in grey consisted of respective duplicated isolates only. Node sizes are according to the numbers of isolates with two being the maximum number of isolates.

The determined cgMLST and the calculated MST are shown in Fig. 3 (a-c). Calculated allelic distances between strains range from 23 to 2181 except for the 14 isolates from duplicated samples in 2018 where the maximum distance is 4 (cluster 1 to 14). Greatest distances were observed between the various MLST's except for the one ST8919 isolate which grouped together with the ST200 isolates (distance to the next ST200 isolate = 75).

When looking at the isolation source of the STEC there is a wide distribution across the German federal states (Fig. 3b). Furthermore, no association between the cgMLST and the matrices from which the isolates originated could be observed (Fig. 3c).

3.5. Comparison of serotype and stx subtype prevalence among flour and clinical isolates

To gain more insights into a possible human health risk posed by the STEC isolates included in this study, strains of selected serotypes were compared to 5370 clinical STEC from the NRC-RKI surveillance strain collection of the same time frame 2015–2019 (Fig. 4). 1105 human isolates were identified for 14 out of the 21 distinct flour serotypes including high prevalent human serotypes O103:H2, O145:H28, O146:H28, O157:H7 and O8:H19. No clusters for human and flour isolates of highly prevalent human serotypes were determined by cgMLST. Distribution of *stx*-gene subtypes in the human strain setting ($n = 1105$) was *stx1a* (13.8%), *stx1c* (10.6%), *stx1d* (0.5%), *stx2a* (25.8%), *stx2b* (23.9%), *stx2c* (14.9%), *stx2d* (2.4%), *stx2e* (6.4%), *stx2f* (2.1%) and *stx2g* (0%). In comparison, in human derived isolates *stx2a* and *stx2b* were most prevalent whereas in isolates from flour and flour products thereof the subtypes *stx1d* and *stx2g* were detected most frequently.

4. Discussion

STEC are zoonotic pathogens which are frequently detected in contaminated ground beef, beef and dairy products (Farrokh et al., 2013; Hussein and Bollinger, 2005; Hussein and Sakuma, 2005). However, recent investigations highlight fresh produce and flour as important source of infection for human outbreaks (Crowe et al., 2017; Feng, 2014; Gill et al., 2019; Kindle et al., 2019; Olaimat and Holley, 2012). In Germany, flour-associated outbreaks have not been reported to date. However, the risk should not be underestimated as Mäde et al. found 39% of their flour samples *stx*-positive. A Federal Surveillance Program in 2018 further confirmed the occurrence of STEC in flour in Germany (https://www.bvl.bund.de).

In our study we analyzed the molecular diversity of 123 STEC isolates from flour, ready-mixes and flour products collected between 2015 and 2019 by official laboratories in Germany including isolates of the FSP. This study not only revealed the occurrence of STEC in flour across Germany, but showed that these STEC are highly diverse in serotype, MLST and the distribution of VAGs. Due to the high genetic diversity a correlation of certain strains to a specific matrix/type of flour or a geographical location could not be determined. This might also be due to the fact that the Federal food inspection laboratories sent their isolates on a voluntary basis to the NRL-*E. coli* and NRC-RKI for further examinations as no legally fixed molecular surveillance exists. Genomic data from the 2018 strains confirmed the high diversity of the STEC strains as 18 different MLST were determined including a new ST10828 for an isolate of serotype O11:H48.

Our analyses of the 123 STEC revealed that strains of serotype O187:H28 were most common, all of which were found to harbour *stx2g*. These strains were isolated between 2015 and 2019 from different matrices which originate from different Federal states indicating a widespread distribution in Germany, rather than a common contamination source. Interestingly, this rare serotype also harbouring the *stx2g* gene was recently reported in a clinical setting in Sweden (Bai et al., 2019). Furthermore, Bai and colleagues identified their isolate as STEC-ETEC hybrid strain of ST200. In our study, 11 of the 12 O187:H28 isolates from 2018 analyzed by WGS were also of ST200 and tested positive for the *est1a/sta1* gene. Strains of O187:H28 ST200 harbouring *stx2g* and *est1a/sta1* genes were also isolated from flour samples in Switzerland (Boss and Hummerjohann, 2019). This highlights the importance of non-O157 and new emerging strains and shows that flour might serve as vehicle for possible human infections.

The second largest group of strains isolated from the samples belong to serogroup O154:H31 all harbouring *stx1d* genes. Similar to STEC O187:H28 these strains were again isolated from different flour types and positive samples were collected from different Federal states. The five isolates from 2018 analyzed by WGS belong to ST1892 but were not positive for any additional VAGs except for F17 fimbriae which might be associated to potential pathogenic *E. coli* (Bihannic et al., 2014).

A previous study from Germany reported the serogroups O157, O91, O26 and O103 as most commonly found in human infections between 1997 and 2013 (Fruth et al., 2015). Among our isolates, we also identified strains of serotypes O157:H7, O103:H2, O145:H28 and O146:H28. WGS analyses of the strains from 2018 showed that especially the O157:H7 and O103:H2 strains harboured high numbers of VAGs including *astA, eae, ehxA/hlyA, esc, espP, nleB,* and *tir* indicating a potential risk to humans of these isolates. Furthermore, strains of O157:H7 and O145:H28 were positive for *stx2c/d* and *stx2a*, respectively. Shiga toxin subtypes 2a and 2d were shown to be more potent for severe clinical symptoms than other subtypes underlining the potential risk of these strains to the consumer (Fuller et al., 2011). The combination of virulence genes *eae* and *nleB* was detected in all *stx1a* –positive O156:H25 isolates as well as the O157:H7 and O145:H28 strains. Contig analyses of the O157:H7 isolate revealed the presence of the large pO157 plasmid containing the *katP, ehxA/hlyA, espP, etpD* and *toxB* virulence genes. This plasmid is highly conserved and frequently found in EHEC O157:H7 isolates (Brunder et al., 1996; Lim et al., 2010; Nielsen and Andersen, 2003) indicating that flour is a potential vehicle for human infections. However, three isolates of O187:H28 were also positive for *katP*. The catalase-peroxidase is proposed to protect pathogens from oxidative stress and it is known that *katP* is not exclusively found on pO157 plasmids (Fan et al., 2019; Gonzalez-Escalona and Kase, 2019; Shridhar et al., 2018).

In our study, STEC of serotype O103:H2 were recovered from the German flour samples. Strains of this serotype are known to cause severe infections in humans in Germany and elsewhere (Fruth et al., 2015; Mathusa et al., 2010), and have been linked to human outbreaks due to contaminated food (Karama et al., 2008; Mylius et al., 2018). Strains of serotype O146:H28 have been previously isolated from game meat, ground beef and faecal samples from deer and cattle (Bosilevac and Koohmaraie, 2011; Dias et al., 2019; Hussein and Bollinger, 2005; Miko et al., 2009) but also from clinical samples (Fierz et al., 2017; Nuesch-Inderbinen et al., 2018). Five isolates were sequenced and showed the presence of the toxin-encoding gene *astA* as well as the VAGs *iha, ireA* and *iss* (increased serum survival) which are known from extraintestinal pathogenic *E. coli* (ExPEC) and uropathogenic *E. coli* (UPEC) (Johnson et al., 2008; Marrs et al., 2005; Russo et al., 2001). In recent studies on STEC in flour in Switzerland, they also found a high diversity of STEC strains and reported isolates of serotypes O103:H2 and O146:H28 (Boss and Hummerjohann, 2019; Kindle et al., 2019). This again shows that

the potential risk to humans should not be underestimated.

In contrast, strains of the serotype O156:H25 might not be listed as one of the top five or six important clinical serotypes. O156:H25 strains have been isolated from cattle, goats and dromedary camels (Barth et al., 2016; Baschera et al., 2019; Blanco et al., 2004; Cortes et al., 2005; Diarra et al., 2009) and have also been isolated from human cases in Germany (Fruth et al., 2015; Lang et al., 2019). The strains from 2018, investigated by WGS, harboured the *stx1a* gene and were assigned as ST300. They also showed high numbers of VAGs including *astA, eae, ehxA/hlyA, esc, espP, etgA, etpD, nleB, paa* and *sep*. STEC of the same serotype and ST were found to be persisting colonizing cattle resulting in a constant shedding of these strains (Barth et al., 2016).

It remains unknown what the main route of contamination of flour and products thereof is but a possible explanation might be the contamination of grain in the field by wild animals (Boss and Hummerjohann, 2019; Mäde et al., 2017). It can be assumed that strains that are able to colonize cattle and other small ruminants may also have the ability to colonize their wild relatives. For O146:H28 strains this is already known (Bosilevac and Koohmaraie, 2011; Dias et al., 2019; Hussein and Bollinger, 2005; Miko et al., 2009). Therefore, continuous monitoring of wild ruminants is needed to gain information about STEC prevalence and distribution of different types which may for example help to initiate control programmes or enable source attribution within outbreak scenarios. In addition to domestic and wild ruminants STEC have been isolated from a wide range of vertebrates, which could potentially serve as the source of initial contamination (Espinosa et al., 2018; Kim et al., 2020). Another contamination source for flour could be contaminated water which is used to temper grain for breaking, or pests at the mills (Mäde et al., 2017). However, neither of these routes has yet been demonstrated as a source of STEC, but a correlation between individual mills and the frequency of STEC isolation indicates that differences in mill process may play a role. (Mäde et al., 2018). Therefore, more data on STEC strains in flour production are needed to determine contamination routes and contribute to the consumers' safety.

Isolation of STEC from flour is challenging (Mäde et al., 2017). In our study, we found that individual samples could be contaminated with more than one STEC strain. Six pairs of strains isolated from a single sample, differed in serotype, *stx*-subtype or both. Therefore, it is important to improve the surveillance on STEC in flour and raise the awareness on rare serotypes and multiple contaminations. The implementation of National surveillance plans are of major importance for systematic evaluation of the risk of contaminated flour on human health. The comparison of STEC serotypes identified in flour and human samples further highlights the possible risk for humans. Most of the serotypes identified were found in both, flour and human samples, including high prevalent clinical strain serotypes but also more rare ones.

This is the first comprehensive overview on the molecular diversity of STEC in flour, ready-mixes and flour products thereof. We determined a high diversity of STEC strains concerning serotypes, MLST and VAG distribution. A correlation to the type of grain or a geographical region was not possible. We identified strains of serotypes associated with cases of human illness, and other strains belonging to uncommon or rare serotypes. However, as the pathogenicity of STEC strains is based on the virulence factors, these rare serotypes should not be ignored as they harboured a high number of VAGs. This is underlined by high numbers of serotype matches between flour and human samples. Furthermore, more than half of the strains from 2018 investigated by WGS were hybrid strains like STEC-ETEC. This in general shows that flour should not be underestimated as source for severe human infections and further investigations are needed to determine contamination and transmission routes of STEC in flour, and flour derived products.

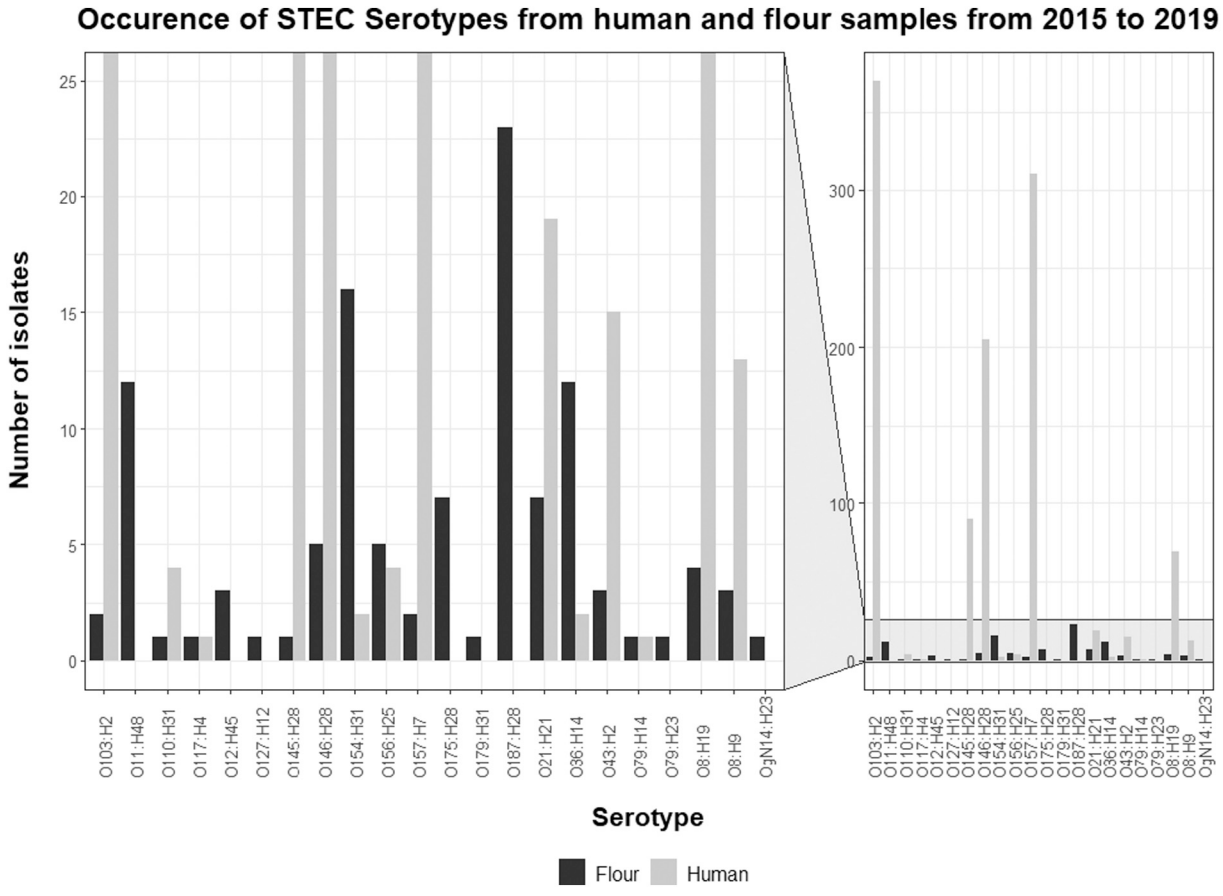


Fig. 4. Distribution of STEC serotypes isolated from flour and human samples between 2015 and 2019 in Germany. OgN: novel O genotype.

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CRediT authorship contribution statement

Michaela Projahn: Conceptualization, Formal analysis, Investigation, Writing - Original Draft & Revision; Marina Lamparter: Conceptualization, Methodology, Investigation, Writing - Review & Editing; Petra Ganas: Formal analysis, Andre Goehler: Data Curation, Sandra C. Lorenz-Wright: Data Curation; Dietrich Maede: Resources; Angelika Fruth: Conceptualization, Resources, Writing - Review & Editing, Christina Lang: Methodology, Investigation, Resources; Elisabeth Schuh: Conceptualization, Data Curation, Project administration, Writing - Review & Editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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