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Virulence and antimicrobial resistance of Shiga toxin-producing *Escherichia coli* from dairy goat and sheep farms in The Netherlands

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Abstract

Aims: The aim of our study was to investigate the virulence and resistance of STEC from small ruminants farms in The Netherlands. Moreover, the potential transmission of STEC between animals and humans on farms was evaluated.

Methods and results: From 182 farms, in total, 287 unique STEC isolates were successfully recovered from animal samples. In addition, STEC was isolated from eight out of 144 human samples. The most detected serotype was O146:H21; however, among other serotypes also O26:H11, O157:H7, and O182:H25 isolates were present. Whole genome sequencing covering all human isolates and 50 of the animal isolates revealed a diversity of stx_1, stx_2, and *eae* sub-types and an additional 57 virulence factors. The assessed antimicrobial resistance phenotype, as determined by microdilution, was concordant with the genetic profiles identified by WGS. WGS also showed that three of the human isolates could be linked to an animal isolate from the same farm.

Conclusions: The obtained STEC isolates showed great diversity in serotype, virulence, and resistance factors. Further analysis by WGS allowed for an in-depth assessment of the virulence and resistance factors present and to determine the relatedness of human and animal isolates.

Significance and impact of study

Our study confirms that farms with small ruminants are a source of a great diversity of STEC. Moreover, possible zoonotic transmission on these farms was observed.

Keywords: virulence, antimicrobial resistance, Shiga toxin-producing *Escherichia coli*, dairy goat and sheep, genetic profiles, WGS

Introduction

Zoonoses, caused by pathogens transmissible from animals to humans, are of great and continuous concern worldwide. In European countries (EEC 1990, EFSA and ECDC 2018), data collection of zoonotic agents in livestock animals and products of animal origin is posed by the European Union (EU) (EEC 1998, 2003). This is also of importance for The Netherlands, as the livestock density and the production of related products, such as meat and dairy, are the highest in comparison to other EU countries (Eurostat 2019). Hence, The Netherlands has implemented a yearly livestock monitoring program at primary production in different sectors. In addition to monitoring the farm animals, farmers, family members, and employees are investigated with the goal to obtain insights in the transmission of relevant zoonotic pathogens to humans on farms (Opsteegh et al. 2018). As part of this monitoring program, dairy goat and sheep farms were studied for the presence of Shiga toxin-producing *Escherichia coli* (STEC) during 2016.

STEC is a causative agent of human gastroenteritis but can also lead to severe diseases like hemolytic uremic syndrome (HUS). Infection can occur by direct contact with livestock or via food. Ruminants and other land animals are considered the main reservoirs for STEC; however, large-scale outbreaks have also been linked to food, such as fresh meat and produce (WHO 2018). In 2016, the year of data collection of our study, of all notifiable zoonotic infections, STEC had the fourth-highest notification rate (1.82 cases per 100 000 population) in Europe (EFSA and ECDC 2017). And a notification rate of 3.92 was reported for The Netherlands in 2016 (EFSA and ECDC 2017). The virulence of STEC is primarily related to the presence of Shiga toxin-encoding genes, stx_1 and/or stx_2, of which a diverse group of variants exists (Scheutz et al. 2012), in addition to other virulence factors like

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the intimin-encoding *eae* gene and the enterohaemolysin *ehxA* determinant. Furthermore, STEC variants are often serotyped, as specific serogroups have been attributed to the disease. The serogroup O157 accounted for the majority of the STEC infections notified in Europe in 2016 (EFSA and ECDC 2017). The main non-O157 serogroups reported to cause illness in Europe with ≥25 cases were O8, O26, O55, O80, O91, O103, O113, O117, O128, O145, O146, and O182 (EFSA and ECDC 2017).

Ruminants like goats and sheep are recognized as a major reservoir of STEC and identified as an important source for human infections using source attribution modeling based on microbial typing data (Mughini-Gras et al. 2018). Other studies in Europe have shown 10.9% of fresh ovine and 11.1% of fresh goat meat to be STEC-positive (EFSA and ECDC 2018). The information available on the prevalence of STEC on farms rearing small ruminants for dairy or meat products is often focused on specific serogroups known to be of clinical relevance in human infections, such as O157 and O26, and these are shown to be present in relatively low abundance (Novotna et al. 2005, Sekse et al. 2011). However, recent reports from EFSA and FAO on STEC virulence indicate all STEC to be potentially pathogenic, showing that broad studies are needed to properly assess the risks (WHO 2018, Koutsoumanis et al. 2020). The limited number of studies available on small ruminants reported a high percentage of STEC for dairy goats and dairy sheep, with a prevalence of ∼50% or more, in Europe and Brazil (Beutin et al. 1993, Zschock et al. 2000, Blanco et al. 2003, Cortes et al. 2005, Oliveira et al. 2008, Schilling et al. 2012). The most common serotypes observed in these studies were O76:H19, O113:H4, O146:H21, and O166:H28. Some of these studies also investigated virulence factors, mainly reporting the intimin gene *eae*, the enterohaemolysin gene *ehxA*, and the enterobactin siderophore receptor gene *iha* (Oliveira et al. 2008, Schilling et al. 2012).

Whole genome sequence (WGS) analysis is becoming an important tool to assess the phylogenetic relationships, virulence, and antimicrobial resistance potential of pathogenic bacteria. By applying WGS tools, the relation between the presence of specific virulence genes can be observed, and the resistance to antibiotics of STEC can be predicted (Ferdous et al. 2016). Moreover, STEC serotypes are more reliably identified by WGS analyses compared to conventional typing methods (Inouye et al. 2014, Joensen et al. 2015, Chattaway et al. 2016).

The 2016 sampling of dairy goat and dairy sheep farms in The Netherlands, as part of our Dutch monitoring program, allowed us to investigate the STEC isolates from the food-producing animals as well as from people working and/or living on these farms in more detail. A selection of obtained isolates was subjected to WGS analysis to extend the knowledge on serotypes, virulence, and antimicrobial resistance profiles for STEC from these farms. The animal-derived and human isolates were subsequently compared in phylogenetic analysis.

Materials and methods

Dairy goat and sheep farms selection

Based on milk-monitoring data from 2014, ∼350 dairy goat and 40 dairy sheep farms were occupationally (>32 animals) active in The Netherlands. Within the Dutch monitoring program, the sample size was determined using Winepi.net to make a statement on the prevalence of the various zoonotic pathogens with a confidence level of 95% (Opsteegh et al. 2018). This resulted in the initial inclusion of 175 randomly selected dairy goat and 35 dairy sheep farms for which contact details were available. In the end, we were able to investigate animal feces from 206 farms: 182 dairy goat and 24 dairy sheep farms.

Screening animal samples

Feces samples were collected throughout the year in 2016 from a total of 206 dairy goat and sheep farms in The Netherlands. From a randomly selected barn of each farm five pooled samples of ∼100 g of animal feces were collected. Each pooled sample consisted of twelve scoops of fresh feces. Additionally, at 78 farms for which the farmer agreed to participate in the human study, between one and five pooled samples were taken from an additional stable. In total, 1141 samples were obtained and analyzed. A cotton swab was used to sub-sample the pooled feces sample, and the swab was subsequently placed in 10 ml modified tryptone soy broth (mTSB) (Thermo Fischer Scientific, Bleiswijk, The Netherlands). The sample was incubated at 37°C for 18–24 h and screened by PCR for the presence of *stx* genes. Bacterial cells were collected by centrifugation of 1 ml of the enrichment and re-suspended in 300 µl of 6% Chelex® DNA extraction suspension (Bio-Rad Laboratories Ltd., Lunteren, The Netherlands). The suspension was incubated at 95°C for 20 minutes and pelleted by centrifugation. The supernatant, containing the DNA, was then transferred to a fresh tube and used for multiplex real-time PCR screening of *stx* genes [ISO/TS 13136:2012 (ISO 2012)].

Isolation and typing of animal-derived isolates

From each farm, one or more positive screening samples were used for STEC isolation. A serial dilution (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) of the enrichment was made with peptone-supplemented (0.1%) saline and spread onto ECC Prisma™ and STEC Colorex™ plates (bioTRADING Benelux B.V., Mijdrecht, The Netherlands). Presumptive positive STEC colonies were streaked onto MacConkey agar plates (BD Difco, The Netherlands), incubated at 37°C overnight, and then stored at 4°C until the PCR results were obtained. The DNA was extracted from isolates by suspending colonies in H_2O and heating for 10 minutes at 95°C in a shaking thermomixer. The multiplex real-time PCR was performed afterward on the DNA isolations to detect stx_1 and stx_2 (stx1/2-F: 5′-TTT GTY ACT GTS ACA GCW GAA GCY TTA CG-3′ and stx1/2-R: 5′-CCC CAG TTC ARW GTR AGR TCM ACR TC-3′, stx1-P: 5′-CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA A-3′ (fluorophore and quencher: FAM-BHQ1) and stx2-P: 5′-TCG TCA GGC ACT GTC TGA AAC TGC TCC-3′ (fluorophore and quencher: HEX-BHQ1) [ISO/TS 13136:2012 (ISO 2012)], and stx_{2f} (stx2f-F: 5′-ATG ACR ACG GAC AGC AGT TAT-3′, stx2f-R: 5′-CTG AAC TCC ATT AAC KCC AGA TA-3′, and stx2f-P: 5′-ATG CAG ATT GGG CGT CAT TCA CTG G-3′ (fluorophore and quencher: Cy5-BHQ3) (Derzelle et al. 2011). The *stx*-positive isolates were streaked onto new MacConkey agar plates and used in BBL crystal enteric/nonfermenter identification analysis (Becton Dickinson Biosciences, Vianen, The Netherlands) to ensure that the isolate was indeed an *E. coli*. Then the isolates were streaked onto

tryptic soy agar (TSA) plates (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) and again confirmed with multiplex real-time PCR for the presence of *stx* (*stx*$_1$ and/or *stx*$_2$). Serotypes were determined for the STEC isolates by the agglutination assay (Friesema et al. 2019). The STEC isolates were stored in cryovials with BHI and 15% glycerol (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) at -80°C for later use.

Screening and isolation of human samples

Human participation was on a voluntary basis using a written informed consent. Out of the 206 farms visited, 144 farmers, employees, and family members from 84 farms sent in a fecal sample (40.8%). This concerned 68 goat and 16 sheep farms. On average, this meant 1.7 participants per farm (range 1–5). The fecal samples were stored together with 10% glycerol/TSB at -70°C prior to testing. Subsequently, 1 g of human feces was enriched in 9 ml BPW (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) overnight at 37°C. The next steps and isolation of STEC were performed according to ISO/TS 13136 (ISO 2012). The obtained STEC isolates were partially serotyped by the EURL-VTEC_Method_03_Rev2, 2020 (Identification of the STEC serogroups mainly associated with human infections by conventional PCR amplification of O-associated genes) published by the European Union Reference Laboratory for *E. coli* available at https://www.iss.it/documents/5430402/0/EURL-VTEC_Method_03_Rev±2.pdf/c9e031a7-8b92-d52b-2e4a-c5848f9a6c80?t=1619466233273.

The human STEC isolates were stored in Microbank™ vials (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) at -70°C and further typed as described below.

Genomic analysis

Fifty representative isolates obtained from animal feces, taking into account their abundance and the diversity of their genetic profiles (serotypes plus the presence/absence of the type of *stx* genes), together with all STEC isolates obtained from human fecal samples were selected for WGS. Each STEC isolate was grown overnight in brain-heart infusion broth (Thermo Fisher Scientific, Breda, The Netherlands) at 37°C. A cell pellet was generated from 1.8 ml of the enrichment and was resuspended in DNA/RNA Shield (Zymo Research, Leiden, The Netherlands). These suspensions were sent to BaseClear (Leiden, The Netherlands) for DNA isolation and WGS analysis. For all isolates, paired-end 2 × 125 bp reads using Nextera XT library preparations (Illumina, Inc) were prepared and sequenced on a HiSeq 2500 platform (Illumina, Inc.). Except for isolate GS51, for which paired-end 2 × 150 bp reads using Nextera XT library preparations (Illumina, Inc) were prepared and sequenced on a NovaSeq platform (Illumina, Inc.). WGS sequences were taxonomically labeled by Kraken to ensure there was no contamination of the DNA samples examined (Wood and Salzberg 2014). Adapter and low-quality sequences (Q <22) were removed by Trimmomatic v0.35 (Bolger et al. 2014). The alignment tool and the *E. coli* database (EcOH) of SRST2 were used for serotyping the isolates (Inouye et al. 2014). Reads were *de novo* assembled by two different tools: ABySS 2.0 (Jackman et al. 2017) and CLC Genomics Workbench 10 (QIAGEN N.V., Venlo, The Netherlands). Both types of assemblies were used to improve the overall detection of virulence and resistance factors. The sequence type (ST) of the STECs was determined by *in silico* seven-locus multi-locus sequence typing by use of the PubMLST database (Jolley et al. 2018). The virulence and antimicrobial resistance genes of *E. coli* were detected by use of VirulenceFinder (Joensen et al. 2014) and ResFinder (Zankari et al. 2012). Genes were recorded as present if 80% of the length of the reference sequences (version of 29 May 2020) was detected with more than 80% identity. The odds ratio was estimated to investigate the association of the presence or absence of a virulence-related gene with the profiles for *stx*$_1$, *stx*$_2$, and *eae*. The statistical significance of the odds ratio was calculated by Fisher's exact test, where the *P*-value was set to 0.05 to reject the hypothesis that the true odds ratio is equal to 1. Single nucleotide polymorphisms (SNPs) were determined for each isolate by mapping the raw reads to the reference genome of NCTC86EC (GCF_900092615.1), using BWA-MEM [version 0.7.15-r1140 (Li 2013)], sorting and deduplicating the resulting alignment using Picard SortSam and MarkDuplicates (https://broadinstitute.github.io/picard), and then calling GATK v3.6 HaplotypeCaller (Poplin et al. 2018) with '—sample_ploidy 1'. Joint genotyping was then performed using GATK v3.6 GenotypeGVCFs. SNPs were excluded as published previously when they had a low coverage (read depth <10), represented conflicting reads (<90% of reads agree with the called genotype), and were found in dense SNP areas (max 3 SNPs per 100 bp) (van den Berg et al. 2019). The filtered SNPs were finally concatenated into SNP profiles. The maximum-likelihood-based phylogenetic tree was inferred for samples by RAxML v8.2.11 (Stamatakis 2014) and visualized by iTOL v5 (Letunic and Bork 2019). The raw read sequences are publicly available under BioProject PRJNA689388 (Supplementary Table S2) at NCBI.

Antimicrobial resistance

The 50 animal-derived isolates selected for WGS analysis were also examined for their antimicrobial resistance with broth microdilution on the standard SENSITITRE EUVSEC panel for *E. coli* containing 14 antibiotics: ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline, and trimethoprim (Thermo Fischer Scientific, Bleiswijk, The Netherlands), conform panels mentioned in Commission Implementing Decision 2013/652/EU. The antimicrobial resistance of isolates for the agents tested was determined using SWIN software (Thermo Fischer Scientific, Bleiswijk, The Netherlands). The European Reference Laboratory (EURL) protocol was followed for the examination (ISO standard 20776–1:2006). Epidemiological cut-off values (ECOFFs) were used for the interpretation of minimum inhibitory concentrations (www.eucast.org was last accessed 10 August 2022).

Results

Prevalence of STEC derived from dairy goat and sheep fecal samples

A total of 1141 pooled fecal samples from 206 selected dairy goat or sheep farms were screened by PCR for STEC in 2016. This included 182 goat farms and 24 sheep farms distributed over The Netherlands. PCR screening of the pooled fecal samples resulted in 1090 (95.5%) positives for *stx*$_1$ and/or *stx*$_2$ (Table 1). Notably, all 206 farms investigated

Table 1. STEC PCR screening results of animal fecal samples collected at dairy goat and dairy sheep farms in The Netherlands.

	Goat farm	Sheep farm	Total
Samples investigated	1006	135	1141
stx positive	961	129	1090
stx_1 only positive	325	16	341
stx_2 only positive	20	9	29
stx_1 and stx_2 positive	616	104	720
Farms investigated	182	24	206
stx positive	182	24	206

were found to be positive for stx_1 and/or stx_2 by PCR screening. In the 1090 *stx*-positive samples, 341 (31.3%) demonstrated the presence of stx_1 and 29 (2.7%) stx_2, while the majority, 720 samples (66.1%) were positive for both stx_1 and stx_2.

Due to the high number of PCR-positive samples, it was impossible to handle STEC isolation for all of them. Therefore, depending on the variety of PCR *stx* profiles detected in the pooled fecal samples per farm, one or two PCR-positive samples were selected for STEC isolation. STEC isolation was performed for 283 samples from the 206 dairy goat and sheep farms included in our study. Overall, STEC isolates were acquired from 220 samples originating from 182 farms. This corresponded to an isolation rate of 77.7% on sample level and 88.3% on farm level. In total, 428 STEC isolates were obtained and stored, since most samples resulted in the isolation of multiple, different STEC isolates. Following isolation, the 428 isolates were checked to confirm the presence of stx_1 and/or stx_2, screened for the presence of the adhesion gene *eae*, as well as serotyped by slide agglutination. In total, 287 unique STEC isolates were obtained, determined by looking at the *stx*/*eae* gene profile and serotype combinations per isolate for each farm (Table 2). Of these, 274 were *eae* negative and 13 *eae* positive. Of the *eae* negative isolates, 157 isolates were stx_1 positive, 30 were stx_2 positive, whereas 87 were stx_1 and stx_2 positive. Of the 13 isolates that were positive for *eae*, seven harbored stx_1, four had stx_2, while two isolates contained both stx_1 and stx_2. All 13 *eae*-positive isolates were obtained from separate farms, eleven from goat farms and two from sheep farms. Serotypes that were identified for ten or more isolates were O146:H21 ($n = 65$), O76:H19 ($n = 32$), O166:H28 ($n = 30$), O113:H4 ($n = 17$), O6:H10 ($n = 11$), and O176:H_{nt} ($n = 10$). Also, isolates from the main serogroups reported to cause illness in Europe (EFSA and ECDC 2017) were identified: O128:H2 ($n = 9$), O182:H25 ($n = 4$), O26:H11 and O26:H_{nt} ($n = 3$), O91:H_{nt} ($n = 1$), O111:H_{nt} ($n = 1$), and O157:H7 ($n = 1$).

Detection of STEC in human fecal samples

Out of the 144 persons from 84 farms (68 goat, 16 sheep) who participated in the human study, eight (5.6%) were found positive for STEC. This concerned persons from seven dairy goat farms and one dairy sheep farm. The STEC isolates were obtained from fecal samples of six farmers, one family member, and one employee, none of which reported any gastroenteric health issues prior to sampling. All human isolates were stx_1 positive and *eae* negative, while five were positive for stx_2. The conventional PCR screening for O-associated genes (EURL-VTEC_Method_03_Rev2, 2020) revealed that four isolates belonged to serotype O146 and one to O113, while the O-type of the other three remained unclear.

Table 2. PCR typing and serotyping results of all STEC isolates derived from dairy goat and dairy sheep feces.

				Total # unique isolates	(% of Total)
Virulence[a]	stx_1	stx_2	*eae*		
	+	+	+	2	(0.7)
	+	+	−	87	(30.3)
	+	−	+	7	(2.4)
	+	−	−	157	(54.7)
	−	+	+	4	(1.4)
	−	+	−	30	(10.5)
Total[b]				287	
Serotyping[c]	O-group	H-type	Serotype		
	O146	H21	O146:H21	65	(22.6)
		H_{nt}	O146:H_{nt}	5	(1.7)
	O76	H19	O76:H19	32	(11.1)
		H21	O76:H21	1	(0.3)
		H_{nt}	O76:H_{nt}	2	(0.7)
	O166	H28	O166:H28	30	(10.5)
	O113	H4	O113:H4	17	(5.9)
		H7	O113:H7	1	(0.3)
		H21	O113:H21	2	(0.7)
		H_{nt}	O113:H_{nt}	1	(0.3)
	O5	H19	O5:H19	6	(2.1)
		H_{nt}	O5:H_{nt}	9	(3.1)
	O176	H4	O176:H4	4	(1.4)
		H_{nt}	O176:H_{nt}	10	(3.5)
	O6	H10	O6:H10	11	(3.8)
	O128	H2	O128:H2	9	(3.1)
	O43	H2	O43:H2	6	(2.1)
		H_{nt}	O43:H_{nt}	1	(0.3)
	O21	H21	O21:H21	6	(2.1)
		H25	O21:H25	1	(0.3)
	O182	H25	O182:H25	4	(1.4)
	O26	H11	O26:H11	2	(0.7)
		H_{nt}	O26:H_{nt}	1	(0.3)
	O91	H_{nt}	O91:H_{nt}	1	(0.3)
	O111	H_{nt}	O111:H_{nt}	1	(0.3)
	O157	H7	O157:H7	1	(0.3)
	Others			58	(20.2)

[a]The number of isolates found to have the virulence genes stx_1, stx_2, and/or *eae* are presented.
[b]The total number of unique isolates with specific serotypes and *stx*/*eae* genes profiles obtained from dairy goat and sheep feces of our study is presented.
[c]The number of isolates of the most abundant and clinically relevant serotypes found in our study is presented. The rest are included as Others in the table. H_{nt}, H-type could not be specified.

Typing STEC isolates using WGS data

All eight human isolates and 50 of the animal-derived isolates were selected for further analysis by WGS (Table 3). Serotypes determined by the WGS serotyping workflow were compared with the results produced by the slide agglutination assay (Supplementary Table S1) and the PCR O-type screening. The serotype from the WGS analysis and the agglutination were identical in 84.0% (42/50) of the animal-derived isolates. For five isolates, the H-type could not be determined by the agglutination assay but was identified by WGS analysis. For two isolates, the O-type was not determined by the agglutination assay, but WGS analysis did show the O-type.

Table 3. Serotypes as well as *stx* and *eae* profiles of the 50 animal-derived isolates selected for WGS analysis.

Serotypes		Virulence factor genes			# Isolates selected for WGS
O-group	H-type	*stx*$_1$	*stx*$_2$	*eae*	
O146	H21	+	+	−	7
	H21	+	−	−	5
	H$_{nt}$	+	+	−	1
O113	H4	+	+	−	2
	H21	−	+	−	2
	H4	+	−	−	1
	H4	−	+	−	1
	H$_{nt}$	+	+	−	1
O76	H19	+	−	−	5
O166	H28	+	−	−	4
	H28	+	+	−	1
O128	H2	−	+	−	2
	H2	+	+	−	1
	H2	+	−	−	1
O5	H$_{nt}$	+	+	−	1
	H$_{nt}$	+	−	+	1
O6	H10	+	−	−	2
O26	H11	+	+	+	2
O182	H25	+	−	+	2
O176	H4	+	−	−	2
O21	H21	+	−	−	1
O91	H$_{nt}$	+	+	−	1
O111	H$_{nt}$	+	−	+	1
O157	H7	−	+	+	1
O$_{nt}$	H14	+	+	−	1
O$_{nt}$	H19	+	−	−	1
Total					50

O$_{nt}$ or H$_{nt}$; the O-group or the H-type were not identifiable as auto-agglutination occurred during the agglutination assay.

For one isolate, the serotype was discordant between the two methods. WGS analysis confirmed the PCR O-type screening results of the human STEC isolates; more specifically, the four O146 isolates were serotyped as O146:H21 and the O113 as O113:H4. The three unknowns were characterized as O5:H19, O21:H21, and O166:H28. All serotypes observed for the eight human STEC isolates were also identified among the small ruminant isolates (Figs. 1 and 2). Moreover, three human isolates showed matching serotypes with an animal-derived STEC from the same farm.

In general, the identified STs of the STEC isolates correlated to a specific serotype (Fig. 1). Although, for two serotypes, WGS analyses showed two STs, that is, ST56 ($n = 1$) and ST223 ($n = 1$) for serotype O113:H21, and ST25 ($n = 2$) and ST811 ($n = 1$) for O128:H2. The three most abundant STs as determined by WGS analysis were ST442 [$n = 13$ (O146:H21)], ST675 [$n = 6$ (O76:H19)], and ST819 [$n = 5$ (O166:H28)]. For serotypes that are clinically relevant in humans, next to, for instance, O113:H21 and O146:H21, the observed STs were ST21 ($n = 2$) for O26:H11, ST33 ($n = 2$) for O91:H14, and ST11 ($n = 1$) for O157:H7.

Profiling of virulence-related genes

The WGS data for 50 animal and eight human-derived STEC isolates were analyzed for the presence of virulence genes using the VirulenceFinder. First, the WGS analysis confirmed the *stx* PCR results, but it also provided *stx* sub-types. For the 43 *stx*$_1$ isolates derived from animal feces, *stx*$_{1a}$ was detected in 8 isolates, while *stx*$_{1c}$ was detected in 35 isolates (Fig. 1, Supplementary Table S2). Notably, all eight human isolates were positive for *stx*$_{1c}$. In total, *stx*$_2$ was found in 30 isolates, with the most identified sub-type being *stx*$_{2b}$, for 23 isolates, of which five had a human origin. Three isolates were positive for *stx*$_{2c}$, two contained *stx*$_{2a}$, while two others harbored *stx*$_{2d}$. The 23 isolates that were positive for both *stx*$_1$ and *stx*$_2$ included 19 with *stx*$_{1c}$ and *stx*$_{2b}$, among which five were isolated from humans. The other combinations were *stx*$_{1a}$ and *stx*$_{2a}$ ($n = 2$), and *stx*$_{1a}$ and *stx*$_{2b}$ ($n = 2$). The seven isolates for which *eae* was detected included O26:H11 ($n = 2$), O182:H25 ($n = 2$), O5:H$_{nt}$ ($n = 1$), O111:H8 ($n = 1$), and O157:H7 ($n = 1$). Four different subtypes of *eae* were encountered, that is, beta1-2, gamma1, theta-1, and zeta1 (for more details see Fig. 1). In addition to *stx*$_1$, *stx*$_2$, and *eae*, 57 other virulence factor genes were detected among the STEC isolates investigated (Fig. 1, Supplementary Table S2). These include genes encoding proteins associated with adhesion, colicin, fimbriae, microcin, the secretion system, serine protease autotransporters of *Enterobacteriaceae*, toxins, and extraintestinal pathogenic *E. coli* (ExPEC)-specific determinants. Two virulence factors were found in all 58 isolates investigated, that is, *gad* and *terC* (Fig. 1). More than half of the isolates investigated were positive for *traT* ($n = 54$), *iss* ($n = 49$), *ehxA* ($n = 46$), *subA* (n = 46), *kpsE* ($n = 44$), *kpsMII* ($n = 44$), *ireA* ($n = 42$), *ompT* ($n = 41$), *lpfA* ($n = 41$), *cia* ($n = 41$), *iha* ($n = 40$), and *senB* ($n = 33$) (Fig. 1). Other interesting virulence genes detected were *astA* ($n = 13$), *tir* ($n = 7$), *toxB* ($n = 2$), and *aaiC* ($n = 2$).

The potential association of the specific virulence genes to *stx*$_1$, *stx*$_2$, or *eae* was determined by the odds ratio and Fisher's exact test for which significance has been indicated in Fig. 1. The presence of the capsule polysaccharide export inner membrane protein-encoding gene *kpsE* and the transport permease protein-encoding gene *kpsMII* were strongly associated with the presence of *stx*$_1$ (Fig. 1). Specifically, 42 out of 51 *stx*$_1$ positive isolates contained *kpsE* and *kpsMII*, whereas only two out of seven *stx*$_1$ negative isolates contained these genes. In contrast, the presence of the colicin-encoding gene (*cea*) was associated with the absence of *stx*$_1$ (Fig. 1). Three genes encoding adhesin (*iha*), aerobactin siderophore biosynthesis protein (*iucC*), and ferric aerobactin receptor (*iutA*), respectively, were associated with the presence of *stx*$_2$, while a protease autotransporter-encoding gene (*pic*) was linked related to the absence of *stx*$_2$. A strict association with *eae* was observed for at least 24 genes (Fig. 1). This includes 14 genes that were mostly present in *eae*-positive isolates ($n = 7$) but were absent in *eae*-negative isolates: *astA* ($n = 7$), *nleB* ($n = 7$), *espJ* ($n = 7$), *espA* ($n = 7$), *tir* ($n = 7$), *nleC* ($n = 6$), *espP* ($n = 5$), *espB* ($n = 4$), *cif* ($n = 4$), *efa1* ($n = 4$), *espF* ($n = 3$), *etpD* ($n = 3$), *katP* ($n = 2$), and *toxB* ($n = 2$). On the other hand, the absence of the *eae* gene ($n = 51$) was strictly associated with the presence of ten genes: *subA* ($n = 46$), *kpsE* ($n = 44$), *kpsMII* ($n = 44$), *ireA* ($n = 42$), *cia* ($n = 41$), *senB* ($n = 33$), *espI* ($n = 24$), *mchB* ($n = 21$), *mchC* ($n = 21$), and *mchF* ($n = 21$).

The three human isolates showing identical serotypes with an animal-derived isolate from the same farm (Fig. 1), displayed closely related virulence profiles. The *stx* and *eae* subtypes matched between the isolates of these pairs. For both the human and animal O113:H4 isolates from farm NL14 (M984 and GS38), the same additional 13 virulence factors were detected. For the O166:H28 isolates from farm NL40, the *sigA* gene, encoding a protein composing the serine protease

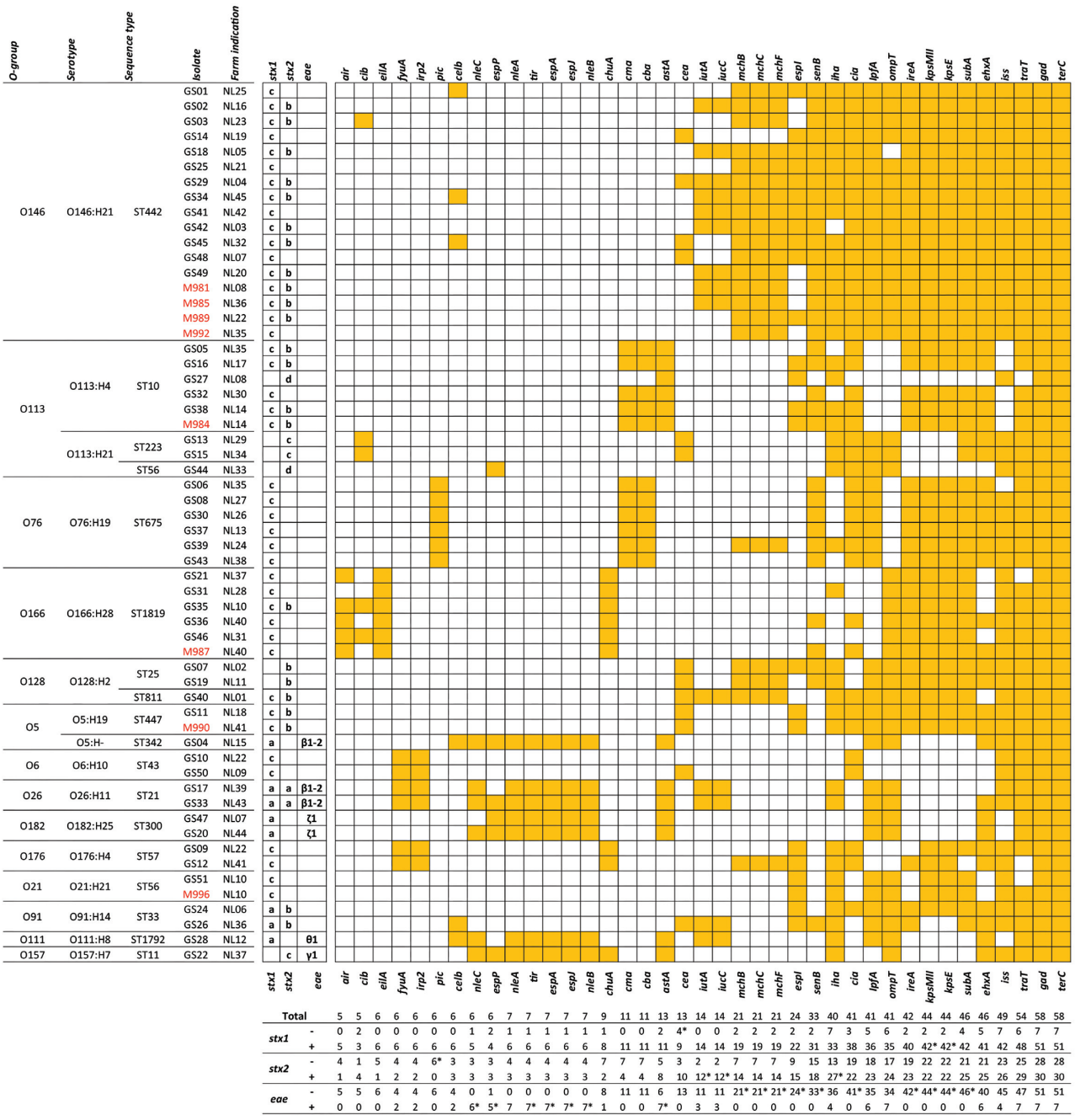

Figure 1. Virulence factors profiles of 58 STEC isolates based on WGS data analysis. The genes detected in five or more isolates are presented. The orange-colored cells indicate that the virulence gene was detected in the isolates [GS (animal) and M (human, in red)] of a specific serotype and ST as determined by the WGS workflow. The blank cells indicate that the gene was absent. The different subtypes of the *stx* genes found are presented in columns *stx1* and *stx2*. The detected *eae* gene is indicated by the identified subtypes: beta1-2 (β1–2), gamma1 (γ1), zeta1 (ζ1), and theta1 (θ1). The number of times a virulence gene was detected is provided at the bottom (total and split based on the presence or absence of stx_1, stx_2, and *eae*). $*$ indicates virulence factors for which sufficient evidence is provided (*P*-value <0.05) to conclude that the presence of the gene was associated with positive or negative detection of stx_1, stx_2, or *eae*.

autotransporters of Enterobacteriaceae, was detected in the human isolate (M987) but not in the animal isolate (GS36). The other additional 15 virulence factors were in agreement. The O21:H21 isolates from location NL10 (M996 and GS51) shared the same profile for eight different virulence factors but showed a discordant profile for *traT*, as this factor was not detected for the animal-derived isolate.

Profiling antimicrobial resistance-related genes of STEC isolates

Antimicrobial resistance phenotypes were predicted for all sequenced isolates based on their ResFinder profiles. Of the 50 animal-derived isolates, six were found to possess at least one antimicrobial resistance gene: GS03, GS04, GS28, GS35, GS46, and GS50 (Table 4). Among these isolates, five

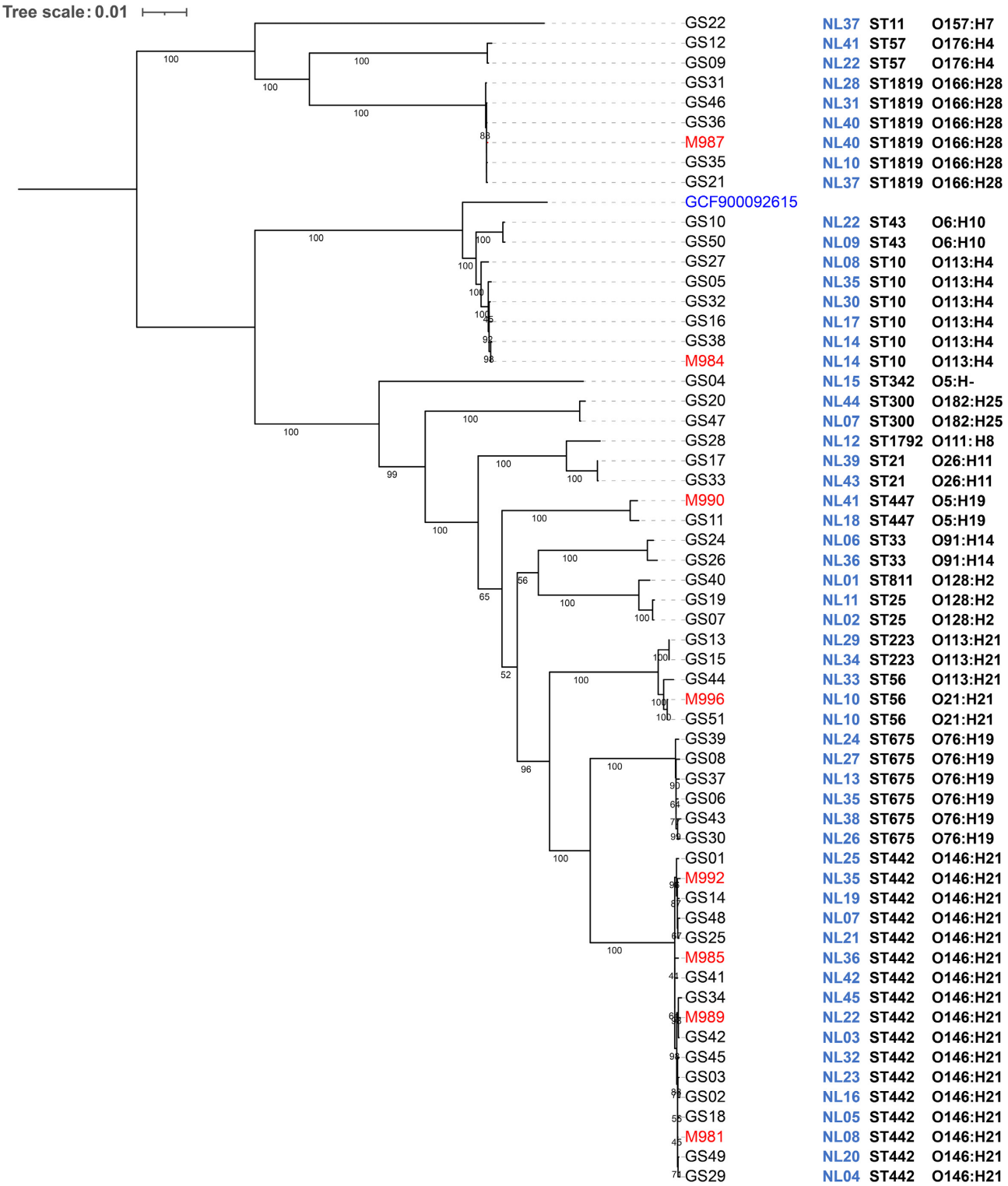

Figure 2. Phylogenetic SNP tree of the isolates derived from humans (M; in red) and animals (GS). The reference genome of NCTC86EC (GCF900092615) is also included in green. The farm IDs (NL), STs, and serotypes are aligned to the isolate IDs.

were predicted as tetracycline-resistant due to the presence of *tet*(A) (GS03, GS28, GS35, GS46, and GS50). Four isolates were putatively sulfonamide and streptomycin-resistant because they contained *sul1* or *sul2* and *strA-strB* (GS04, GS28, GS35, and GS50). Two isolates harbored $bla_{\text{TEM-1b}}$ making them possibly ampicillin-resistant (GS28 and GS50). Two others with *aadA1* were potentially streptomycin and spectinomycin-resistant (GS35 and GS50). One fosfomycin-resistant isolate (GS04 with *fosA7*) and one trimethoprim-resistant isolate (GS50 with *drfA1*) were also predicted. Except for three substances that were not tested in the phenotypic panel (i.e. fosfomycin, spectinomycin, and streptomycin), the phenotypic antimicrobial resistance profiles were fully in accordance with the genetic profiles. Exploring antimicrobial

Table 4. Antimicrobial resistance of STEC isolates.

		Predicted phenotype[b]							Confirmed phenotype[c]						
Isolate	AMR genes detected[a]	AMP	FOS	SMX	SPT	STR	TET	TMP	AMP	FOS*	SMX	SPT*	STR*	TET	TMP
GS03	*tet*(A)														
GS04	*fosA7, sul2, strA-strB*		x	x		x					x				
GS28	$bla_{\text{TEM-1b}}$, *sul2, strA-strB, tet*(A)	x		x		x	x		x		x			x	
GS35	*sul1, aadA1, tet*(A)			x	x	x	x				x			x	
GS46	*tet*(A)						x							x	
GS50	$bla_{\text{TEM-1b}}$, *sul1, sul2, aadA1, strA-strB, tet*(A), *dfrA1*	x		x	x	x	x	x	x		x			x	x*

[a]The ResFinder profiles of antimicrobial resistance (AMR)-related genes detected by the ResFinder workflow of WGS. AMP, ampicillin; FOS, fosfomycin; SMX, sulfamethoxazole; SPT, spectinomycin; STR, streptomycin; TET, tetracycline; TMP, trimethoprim.
[b]x marks the predicted phenotypes on antimicrobial resistance based on the ResFinder profile (AMR genes detected).
[c]x marks the confirmed phenotypes on antimicrobial resistance as determined with broth microdilution. Antimicrobial resistance was tested against 14 antibiotics; included were AMP, ampicillin; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; CTZ, ceftazidime; GEN, gentamicin; MER, meropenem; NDA, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim.
Antibiotics not assessed by the defined method are indicated by an asterisk (*).

resistance phenotypes at the isolate level, this identified two isolates being resistant against one antibiotic only [tetracycline (GS03 and GS46)]; one resistant against three antibiotics [fosfomycin, sulfamethoxazole, and streptomycin (GS04)] two resistant against four antibiotics [sulfamethoxazole, spectinomycin, streptomycin, and tetracycline (GS28); ampicillin, sulfamethoxazole, streptomycin, and tetracycline (GS35)]; and one isolate resistant against six antibiotics [ampicillin, sulfamethoxazole, spectinomycin, streptomycin, tetracycline, and trimethoprim (GS50)]. Among the human isolates, no antimicrobial resistance genes were detected; therefore, these were not tested phenotypically.

Phylogenetic analysis comparing human and animal isolates

Phylogenetic analysis to investigate human and animal-derived STEC relatedness showed grouping according to the STs of the isolates and, to a lesser extent, their serotypes (Fig. 2). For example, GS44 (O113:H21) clustered closer to two O21:H21 isolates than to the other two O113:H21 STECs. This is probably due to the fact that the ST of GS44 is identical to the O21:H21 STECs (ST56) and different from the other O113:H21 isolates (ST223). The eight human isolates included in the phylogenetic analysis clustered among the animal isolates with identical STs. The largest SNP difference between a human-derived isolate and its most closely related animal-derived isolate was 39 (M990 (farm NL41) and GS11 (farm NL18). In comparison, all other SNP differences between a human isolate and its closest animal isolate were below 15 SNPs. Three human isolates [M984 (farm NL14), M987 (farm NL40), and M996 (farm NL10)] were closely related to animal-derived isolates from these same farms, with only 2–4 SNPs observed. In addition to the short SNP distance, bootstrap values confirmed the relatedness of these isolates. M984 clusters together with five other O113:H4 isolates, of which isolate GS38 clusters the closest to M984 with only two SNPs and a bootstrap support of 98. M987 was part of a group of six isolates of O166:H28. And although isolate GS35 (NL40) clusters closest to M987 from the same farm (four SNPs), they are both part of a bigger group containing the six O166:H28 isolates, for which the bootstrap support is 100. Clustering of separate O166:H28 isolates within this group resulted in a bootstrap support lower than 90. The biggest distance within this O166:H28 group is seven SNPs. M996 clusters together with the only other O21:H21 isolate GS51, with 2 SNPs and a bootstrap support of 100.

Discussion

The initial *stx* PCR screening in our study showed the presence of *stx* genes at all 206 investigated dairy goat and dairy sheep farms in The Netherlands in 2016. Overall, 95.5% of the goat farm samples investigated and 95.6% of the sheep farm samples were *stx* positive in the screening of the enrichment cultures (Table 1). This corresponds with previous reports showing that small ruminants are an important reservoir for STEC (Beutin et al. 1993, Zschock et al. 2000, Oliveira et al. 2008, EFSA and ECDC 2018). The *stx* PCR screening is not absolute for the presence of STEC, due to the possible presence of free Shiga toxin-encoding bacteriophages, or the presence of *stx* genes in species other than *E. coli* (Koutsoumanis et al. 2020). Therefore, the identification of STEC requires culture enrichment, although this is not always possible in cases where STEC is present in low concentrations, when cells are not in a culturable state, or when commensal *E. coli* interferes with isolation. Our study resulted in the actual isolation of STEC isolates from the selected samples ($n = 283$) with all farms represented in an isolation rate of 77.7% at the sample level and 88.3% at the farm level.

Thus, the average prevalence of STEC (*stx*-positive samples) in farms with healthy small ruminants in The Netherlands in 2016 was higher than similar studies in other countries, which reported a lower prevalence of between 56% and 76% for dairy goats and 32% and 67% for dairy sheep in Brazil and Germany (Beutin et al. 1993, Zschock et al. 2000, Oliveira et al. 2008). These differences might be explained by differences between countries, but can also be due to technical differences between the studies, such as a different sampling scheme or the use of different culture media and agar plates. For instance, the use of chromogenic agar plates in this study could have increased the isolation chance.

Our study also determined the number of positives in the PCR screening of the enrichment step for stx_1 (29.9%), stx_2 (2.5%), and for both stx_1 and stx_2 (63.1%) (Table 1). Focusing on the STEC isolates themselves, however, a much higher proportion of stx_1 positive isolates was recovered: 57.1% ($n = 164$), versus stx_2 positives 11.8% ($n = 34$), and stx_1 and stx_2 positives 31.0% ($n = 89$) (Table 2). This can be explained by the fact that free bacteriophages are present, or by the fact that STEC strains with either *stx1* or *stx2* were both present in the samples PCR positive for both stx_1 and stx_2. Both these facts can result in an altered distribution of *stx* genes between the screening results and the isolates. As far as known there are no technical details in the study design leading to the preferred isolation of any of the *stx* genes. Also, previous studies in Germany and Brazil reported the prevalence of stx_1 positive, stx_2 positive, and $stx_1 + stx_2$ positive *E. coli* to be greatly diverse, with respective percentages of 2.5%–98.6%, 15.0%–49.2%, and 1.4%–72.5% in goat fecal samples and 21.2%–66.7%, 26.5%–33.3%, and 52.2% in sheep feces (Beutin et al. 1993, Zschock et al. 2000, Oliveira et al. 2008).

The most abundant O-types of STEC found for dairy goat and sheep farms in The Netherlands were O146 with 24.3% ($n = 70$) of the isolates, followed by O76 with 12.1% ($n = 35$) and O166 with 10.5% ($n = 30$) (Table 2). O146:H21 is a commonly observed serotype in small ruminants; as are O76:H19, O113:H4, and O166:H28 (Oliveira et al. 2008, Schilling et al. 2012). While our study detected O91 and O166 solely in goat samples, previous studies detected STEC isolates of the serotypes O91 ($O91:H_{nt}$) and O166 (O166:H28) from sheep or lamb in Germany and Spain (Beutin et al. 1993, Blanco et al. 2003).

The WGS serotyping workflow successfully identified the O- and H-types of 49 animal-derived isolates (98.0%) but failed to characterize the H-type of one isolate (GS04) (Table 3, Supplementary Table S1). In contrast, for seven isolates, the O- and/or H-type could not be identified by the agglutination assay. This confirms that agglutination assays are less conclusive than WGS analysis, as also stated previously (Chattaway et al. 2016). One of three isolates of both O113:H21 and O128:H2 was assigned to a different ST. Both serogroups are known to consist of different closely related STs, including ST56 and ST223 for O113:H21 (Monaghan et al. 2012) and ST25 and ST811 for O128:H2 (Joensen et al. 2014).

Besides *stx* genes and *eae*, 57 additional virulence genes were identified for the 58 STEC isolates studied by WGS (Fig. 1). More than half of the isolates possessed *cia*, *ehxA*, *iha*, *ireA*, *iss*, *kpsE*, *kpsMII*, *lpfA*, *ompT*, *senB*, *subA*, and *traT*. Other interesting genes in relation to virulence that were detected are *astA* ($n = 13$), *toxB* ($n = 2$), *tir* ($n = 7$), and *aaiC* ($n = 2$) (Fig. 1, Supplementary Table S2). The enterohaemolysin gene *ehxA* has been detected previously in STEC isolated from caprine and ovine animals (Blanco et al. 2003, Oliveira et al. 2008). However, virulence genes such as the autoagglutinating adhesion encoding determinant (*saa*) and the cytotoxic necrotizing factor (*cnf1*), prevalent in STEC obtained from rectal swabs of sheep investigated in Brazil (Ferreira et al. 2015), were not detected in our study. Moreover, for 20 virulence factors that were found to be associated with the presence or absence of *eae*, the same association was identified previously for clinical isolates in The Netherlands (Ferdous et al. 2016). The associations of these genes with *eae* are not unexpected, as for instance, *eae* is located together with *espA*, *espB*, *espF*, and *tir* on a 35.6 kb pathogenicity island, the locus of enterocyte effacement (LEE) (Franzin and Sircili 2015). Moreover, *espP*, *etpD*, *katP*, and *toxB* are located on the virulence plasmid pO157, which is more common among *eae*-positive isolates. (Cavalcanti et al. 2020). In addition, *espJ*, *nleB*, and *nleC* are part of non-LEE pathogenicity islands (Dean and Kenny 2009), and these genes also have a positive association with *eae*, as reported previously (Creuzburg et al. 2011, Ferdous et al. 2016). For the genes *cia*, *kpsE*, *kpsMII*, and *subA* that were present significantly more in *eae*-negative samples in our study, associations have been described before. For instance, for *subA* from STEC obtained from cattle and ovine samples, a negative association with *eae* has been reported (Michelacci et al. 2013, Caceres et al. 2017). The colicin gene *cia* was previously detected in a diversity of O91 strains, which were all negative for *eae* (Nuesch-Inderbinen et al. 2021). In addition, the *kps* genes (*kpsMII* and *kpsE*) are associated with ExPEC strains (Olesen 2017, Nuesch-Inderbinen et al. 2021). This explains the fact that these *kps* genes are not linked with the presence of *eae*, as intimin has a role in binding to the intestine.

The antimicrobial resistance of the isolates was assessed to add information to the long-term monitoring program for clinical STEC isolates, which is reported yearly for The Netherlands in the MARAN report. However, for goats and sheep, only limited data is available for The Netherlands. In our study, out of the 50 animal-derived isolates tested, only six were identified to contain antimicrobial resistance genes and to show the corresponding phenotype (Table 4). All six of these isolates were obtained from goat feces. Antibiotic-resistant isolates of STEC have been tested in sheep before (Ferreira et al. 2015, Amezquita-Lopez et al. 2016), and a high prevalence was shown (83.3% of the isolates) (Ferreira et al. 2015), but antibiotic resistance has been less often tested and described for goat (Novotna et al. 2005). Moreover, when comparing resistance profiles between countries, it should be considered that the use of antimicrobials between countries can differ significantly, hence causing great differences in resistance profiles. The most prevalent resistance profiles observed in our study, that is, ampicillin, sulfamethoxazole, and tetracycline resistance, were also previously reported for sheep (Ferreira et al. 2015, Amezquita-Lopez et al. 2016). For the other antibiotics (fosfomycin, spectinomycin, and streptomycin), only genetic data were obtained in our study, and comparative analyses were not available. Remarkably, all isolates with an observed resistance contained the stx_1 gene, in two cases in combination with stx_2. The tendency of high prevalence of resistance for stx_1-positive isolates was also observed for antibiotic-resistant STEC from Dutch patients (Ferdous et al. 2016), although for none of the eight human isolates from this study antibiotic resistance genes were observed.

Recent international reports indicate all STEC to be potentially pathogenic (Koutsoumanis et al. 2020). However, STECs with specific combinations of virulence factors are still considered the most potent to cause disease. The WHO suggests a five-level classification to assess STEC pathogenicity, rather than relying on the STEC serotype (WHO 2018). In our study, two isolates belonged to the highest level of pathogenicity (stx_{2a} and *eae;* O26:H11), which means these isolates are more prone to cause HUS, or hemolytic colitis. One isolate belonged to the second-highest level (level 2) of pathogenicity (stx_{2d}; O113:H4), one isolate to level 3 (stx_{2c} and *eae*; O157:H7), and four isolates to level 4 (stx_{1a} and *eae*; 2x O182:H25, 1x O111:H8, 1x $O5:H_{nt}$). All other

isolates, including all of the eight human isolates, are considered level 5 STEC (at least *stx* present). Moreover, based on their serotypes, 70% (35/50) of the animal-derived isolates investigated by WGS in our study were also identified as serotypes causing human infections in Europe (EFSA and ECDC 2018).

The combination of monitoring both human and animal samples in our study generated unique insights into the transmission possibility of STEC from small ruminants to humans on these farms. The isolates obtained from human samples were closely related to the STEC obtained from animal-derived samples, and even more so when both the human and animal isolates were obtained from the same farm (Fig. 2). These pairs of isolates from the same farm also showed a match in virulence factors (Fig. 1), although for two virulence genes, their presence was only observed for the human isolate. These concerned *sigA* for human isolate M987 and *traT* for M996 compared to animal isolate GS36 and GS51, respectively, for which these genes were absent. Since *sigA* was only detected in the human isolate M987, it was therefore not presented in Fig. 1. To determine if these differences could be due to minor variances in the assemblies of the genomes, the raw reads of the corresponding animal isolates were mapped on the assemblies of the human isolates. No reads were detected to map, respectively, for *sigA* and *traT* indicating that these genes were truly absent in the animal isolates. The difference could be the result of the undersampling of related STEC variants existing on the sampled farms or because of horizontal gene transfer occurring in the human gut. For *traT*, it is known that it is generally located on a plasmid (Achtman et al. 1977). This can be the cause of either the loss of *traT* in the animal isolate or the gain of the mobile genetic element with this gene in the human isolate.

The closely related STEC isolates, obtained from human and small ruminant samples from the same farm, suggests transmission from the animal reservoir to humans on these farms. None of the human participants that were STEC-positive reported any symptoms related to a possible STEC infection. Studies on STEC in human samples related to small ruminant farms are scarce, especially regarding healthy adults. Morita-Ishihara *et al.* (2016) described a prevalence of asymptomatic carriers among healthy adults of 0.08% (398/472.734) in Japan, and Urdahl et al. (2013) found one STEC isolate in 165 volunteers (0.6%) in Norway. Both these percentages are far lower than the 5.6% encountered in our study, which is probably due to the high exposure with STEC-infected colonized animals on the Dutch small ruminant farms. The single STEC isolate from the Norwegian study was typed as O146:H21, harboring stx_{1c} and stx_{2d}. This combination of serotype and *stx* genes was also the most abundant combination among the human participants of our study. In addition, in The Netherlands, it is a serotype of clinical importance attributable to small ruminants (Mughini-Gras et al. 2018). Most investigations regarding asymptomatic adult human STEC carriers have focused on humans working in the meat processing industry, but often only describing prevalence numbers based on *stx* PCR screening data. For example, studies from Switzerland reported a prevalence of 4.6% and 3.5% of *stx*-positive stools among 1730, and 5590 tested individuals (Stephan and Untermann 1999, Stephan et al. 2000). In addition, Hong et al. (2009) reports a prevalence of 5.6% based on 1602 individuals in South Korea. Although STEC isolates were obtained in these three studies, unfortunately prevalence data cannot be deduced from the published results.

In conclusion, STEC isolates derived from dairy goat and dairy sheep farms in The Netherlands showed a high prevalence and great diversity of these pathogens in this small ruminant reservoir. Applying a WGS-based analysis allowed for a more in-depth profiling of these isolates. The potential pathogenicity and antimicrobial resistance of the STEC isolates were analyzed and it was shown that only a limited number of highly pathogenic STEC were observed in the small ruminants. Moreover, the prevalence of STEC carriage in asymptomatic adults living and/or working on the dairy goat and dairy sheep farms was high compared to the general population, and zoonotic transmission of STEC was probable on three farms where closely related WGS profiles in animal and human strains were identified. For this reason, and taking into account the resilience and flexibility of *E. coli*, it is relevant to keep monitoring small ruminants for the presence of highly pathogenic STEC.

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

The authors declare to have no conflict of interest.

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Data availability

The data underlying this article are available in the article and in its online supplementary material. Moreover, whole genome sequencing data are available in NCBI BioProject PRJNA689388.

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