Microbiology and Immunology

Microbiol Immunol 2015; 59: 433–442
doi: 10.1111/1348-0421.12275

ORIGINAL ARTICLE

Detection of virulence-associated genes characteristic of intestinal *Escherichia coli* pathotypes, including the enterohemorrhagic/enteroaggregative O104:H4, in bovines from Germany and Spain

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ABSTRACT

Cattle are reservoirs of enterohemorrhagic *Escherichia* coli; however, their role in the epidemiology of other pathogenic *E. coli* remains undefined. A new set of quantitative real-time PCR assays for the direct detection and quantification of nine virulence-associated genes (VAGs) characteristic of the most important human *E. coli* pathotypes and four serotype-related genes (wzx_{O104},$fliC_{H4}$, rbf_{O157},$fliC_{H7}$) that can be used as a surveillance tool for detection of pathogenic strains was developed. A total of 970 cattle fecal samples were collected in slaughterhouses in Germany and Spain, pooled into 134 samples and analyzed with this tool. *stx*1, *eae* and *inv*A were more prevalent in Spanish samples whereas *bfp*A, *stx*2, *ehx*A, *elt*, *est* and the rbf_{O157}/$fliC_{H7}$ combination were observed in similar proportions in both countries. Genes characteristic of the hybrid O104:H4 strain of the 2011 German outbreak (*stx*2/*agg*R/wzx_{O104}/$fliC_{H4}$) were simultaneously detected in six fecal pools from one German abattoir located near the outbreak epicenter. Although no isolate harboring the full *stx*2/*agg*R/wzx_{O104}/$fliC_{H4}$ combination was cultured, sequencing of the *agg*R positive PCR products revealed 100% homology to the *agg*R from the outbreak strain. Concomitant detection by this direct approach of VAGs from a novel human pathogenic *E. coli* strain in cattle samples implies that the *E. coli* gene pool in these animals can be implicated in *de novo* formation of such highly-virulent strains. The application of this set of qPCRs in surveillance studies could be an efficient early-warning tool for the emergence of zoonotic *E. coli* in livestock.

Key words Escherichia coli, virulence-associated genes, pathotypes, O104:H4.

Escherichia coli are facultative anaerobic bacteria that exist in the intestines of warm-blooded mammals, including humans. Many *E. coli* strains are nonpathogenic and are commensals in their natural hosts. However, some strains can cause intestinal disease, producing in the most severe cases hemorrhagic

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Received 22 April 2015; revised 8 June 2015; accepted 12 June 2015.

List of Abbreviations: cPCR, conventional PCR; DIG, digoxigenin; EAEC, enteroaggregative *E. coli*; EFSA, European Food Safety Authority; EHEC, enterohemorragic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; HUS, hemolytic uremic syndrome; qPCR, quantitative PCR; STEC, shigatoxin-producing *E. coli*; VAG, virulence associated gene.

colitis and Hemolitic Uremic Syndrome (HUS). These pathogenic *E. coli* are grouped into ETEC, EPEC, STEC (including the subset known as EHEC), EAEC and EIEC (1). The pathogenicity of *E. coli* is closely linked to the carriage and expression of certain VAGs and combinations thereof (2).

In 2011, a novel EHEC/EAEC hybrid strain of serotype O104:H4 emerged in Germany and neighboring countries, causing 2987 cases of gastroenteritis and 855 of HUS, and leading to the deaths of 54 patients (3). This strain possessed VAGs from two different *E. coli* pathotypes (EAEC: *agg*R; EHEC: *stx*2) in a unique combination rarely previously detected. Although its exact natural reservoir remains unknown, contaminated fenugreek sprouts were the suspected source of the outbreak (4). The most fearsome aspect of this outbreak is that recombination events within the *E. coli* gene pool present in a given habitat may again lead to generation of an EHEC strain with similar virulence traits; hence, similar outbreaks may occur again (5). Lessons from this episode argue strongly in favor of improved surveillance of *E. coli* VAGs in habitats that may act as sources of contamination in the food chain, including the use of cost-effective, rapid, specific, cutting edge, diagnostic tools (5, 6).

In the case of pathogenic *E. coli*, most of the current methodologies for monitoring in animals follow a culture-based approach and subsequent molecular testing (7–9). The fact that only a small proportion of *E. coli* colonizing a particular animal may be recovered and further identified, thus increasing the probability of false negative results, limits the ability to detect pathogenic bacteria. In contrast, qPCR directly targeting VAGs in fecal samples provides a superior approach (10, 11), allowing not only qualitative (i.e., presence/absence of a particular VAG) but also quantitative detection of particular molecular targets and combinations thereof (such as a set of VAGs). A screening protocol based on this approach could provide the basis for a more sound assessment of the risks associated with a certain habitat and also support epidemiological investigation when the causative agent of a suspected source has not been isolated.

Here, we present a surveillance study based on new real-time qPCR assays for the detection of nine *E. coli* VAGs belonging to *E. coli* intestinal pathotypes, as well as four marker genes for two serotypes of Public Health importance (O157:H7 and O104:H4). We evaluated these qPCRs in DNA directly extracted from pooled feces of healthy cattle slaughtered in different abattoirs in Germany and Spain to define herd-/region-/country-specific differences in the prevalence of the targeted VAGs.

MATERIALS AND METHODS

Field sample collection

A total of 970 fecal samples from cattle were collected in slaughterhouses in Germany and Spain. In Spain, abattoirs were selected depending on their slaughter loads, the whole selection representing at least 50% of the national slaughter capacity. In addition, slaughterhouses were selected to include at least 50% of the Autonomous Regions. Feces from 66 different farms (330 individual samples) were collected at 15 slaughterhouses between June and July 2012. In Germany, 68 different farms (640 individual samples) were sampled at six slaughter houses from July to December 2012 (Fig. 1). For each Spanish farm, feces from five different cows (three grams per cow) arriving at the slaughterhouse in the same batch from the same feedlot were collected and immediately submitted to the laboratory and processed within the following 24 hr or frozen at −40°C until submission. In German abattoirs, fecal samples from one to five cows per farm were collected, sent refrigerated to the laboratory and stored at −80°C. All five samples from the same feedlot (Spanish samples) and one to five samples from each feedlot (German samples) were pooled and diluted 1:2 with sterile PBS to create a total of 134 pooled samples, after which 400 mg of each pool were subjected to DNA extraction and purification with a commercial DNA kit (QIAamp DNA stool mini-kit; Qiagen, Heiden, Germany).

Primer design and qPCR analysis

Nine VAGs typically linked to five intestinal *E. coli* pathotypes (EHEC: *stx*1, *stx*2, *ehx*A, *eae*; EAEC: *agg*R; ETEC: *est*, *elt*; EPEC: *ehx*A, *bfp*A, *eae*; EIEC: *inv*A) and four serotype-related genes (somatic and flagellar antigens wzx_{O104}/rbf_{O157} and $fliC_{H4}$/$fliC_{H7}$, respectively) were selected as targets for eight qPCRs to test the 134 pooled samples.

Primer pairs used in the qPCRs were previously described in our conventional PCR (7). Taqman probes for these amplicons and primer pairs for generation of the standard curves products were designed using Oligo software (Oligo primer analysis software v7, Cascade) (Tables S1 and S2). The DNA sequences of each gene were assembled from GenBank (12). Their sequences were checked against several known microbial genomes other than *E. coli* using the BLAST N tool (Basic Local Alignment Search Tool; National Center for Biotechnology Information, Bethesda, Maryland, USA) to verify that there were no unspecific matches.

Several *E. coli* reference strains were used as positive controls for the qPCR and to generate PCR amplicons

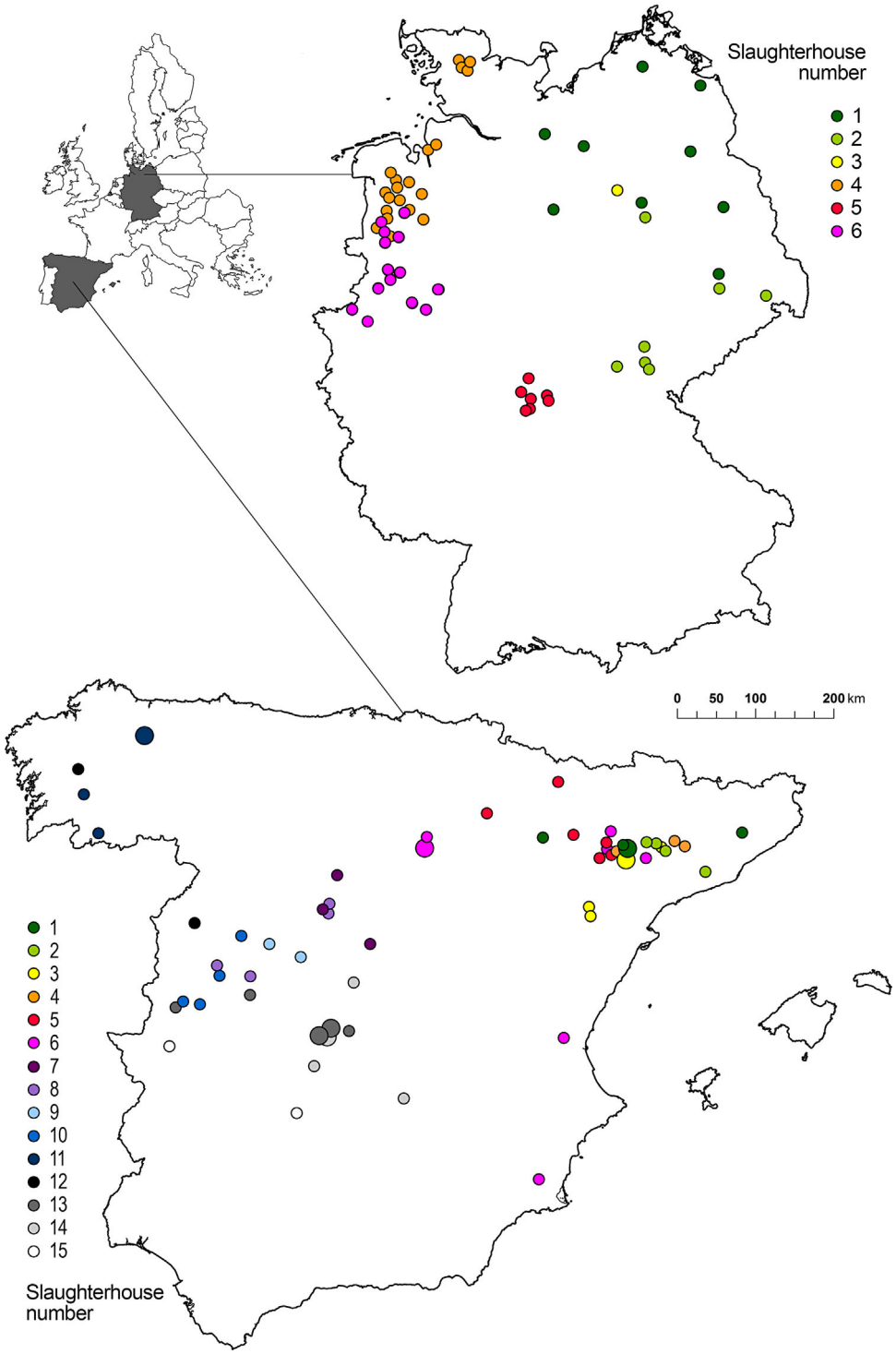

Fig. 1. Locations of the sampled farms in Germany and Spain. Each circle represents a single farm. The sizes of the circles indicate the number of sampled farms per country. Cattle from all farms of the same color were sent to the same slaughterhouse.

for the construction of the standard curves needed for the quantitative analysis. These strains were as follows: a STEC O157:H7 (strain CNM 2686/03 positive to *stx*1/*sxt*2/*eae*/*ehx*A); a typical EPEC (strain CNM 764 positive to *bfp*A/*eae*), kindly provided by Dr. Silvia Herrera-León (National Center of Microbiology, Institute of Health Carlos III, Madrid, Spain); four ETEC (strains 117–86 [HYMS007], H10407, H297/89 and G1253 positive to *elt* and/or *est*); two EIEC (strains 1280 and 76–5 [HYMS006] positive to *Inv*A); an O104:H4 EAEC (strain 55989 positive to *agg*R); and the EAEC/EHEC outbreak strain (LB226692 positive to *agg*R/*sxt*2), all of which were provided by Dr. Martina Bielazweska (Institute of Hygiene, Münster University, Germany).

Given that wzx_{O104}, $fliC_{H4}$, rbf_{O157} and $fliC_{H7}$ are chromosomal single copy genes, 10-fold dilutions from one of the O104:H4 and O157:H7 strains were prepared to generate real-time standards for these four targets (wzx_{O104}, $fliC_{H4}$, rbf_{O157}, $fliC_{H7}$). As the gene copy numbers of most of the VAGs were unknown because they were encoded in plasmids, in order to make the results comparable with that of the serotype-related genes, a second primer pair targeting a wide region of each VAG of interest (and comprising the sequence of the qPCR primers and the probe) was used to generate standard curves for each VAG. Primers were used in a cPCR assay (10 μL of MasterMix [Qiagen], 6.5 μL of bi-distilled water and 2 μL of a mix of primers 200 nM), their products purified and their optical densities measured with NanoDrop. DNA concentration was adjusted to 1 ng/μL and serially 10-fold diluted with double-distilled water.

The specificity of the qPCR reactions was checked by testing the above mentioned positive control strains and the following negative control strains: an *E. coli* ATCC 25922 (CECT 434) that did not harbor any of the targeted virulence factors; a *Salmonella enterica* subsp. *enterica* serovar enteritidis CECT 4300; a *Salmonella enterica* subsp. *enterica* serovar typhimurium ATCC 14028 (CECT 4594); an *Enterococcus faecalis* ATCC 29212 (CECT 795); a *Campylobacter jejuni* ATCC 33560, a *Staphylococcus aureus* ATCC 29213 (CECT 15915); and a *Staphylococcus aureus subsp. Aureus* ATCC 25923 (CECT 435). The qPCRs for the 134 pooled samples were performed as both simple and multiplex assays using newly designed TaqMan probes. The selected gene combinations were as follows according to the melting temperature of the primers: *stx*1/*stx*2, *agg*R, wzx_{O104}/$fliC_{H4}$, rbf_{O157}/$fliC_{H7}$, *Inv*A/*bfp*A, *eae*, *est*/*elt* and *ehx*A. The melting temperature for each primer was calculated with Oligo software (v.7) and then optimized by repeating each reaction by using a thermal gradient from 50 to 65°C. qPCR was carried out in a final 25 μL volume containing 12.5 μL of a qPCR master mix (QuantiTect Multiplex PCR NoROX Kit cat. no. 204743; Qiagen), and 1.25 μL of each mix of the corresponding primers (Invitrogen Life Technologies, Carlsbad, CA, USA) and probes (Eurofins Genomics GmbH, Ebersbach, Germany) and then adjusted with double bi-distilled water. In the qPCR, the work concentrations were 500 nM for the primers and 200 nM for the probes. Finally, 5 μL of the different DNA templates were added. Cts of each target were compared in single and multiplex assays to ensure that no combination of primers and probes adversely affected the amplification of any of the targets. Three replicates were performed for each sample and mean values were obtained. PCRs were conducted using an iCycler Thermal cycler (Biorad Laboratories, Hercules, CA, USA). Amplification conditions were: 95°C for 15 min followed by 40–45 cycles of 1 min at 94°C, 1 min at 52–55°C and a final extension step of 10 min at 72°C. Standards and samples were tested in triplicate and data were checked using iCycler iQ v.3.1 software. Efficiencies out of the 90–110% range were regarded as unacceptable in accordance with the manufacturer's recommendations. Each VAG is expressed as number of copies of each gene per mg of feces, taking the initial dilution factor into account. All reactions included a positive control (one reference strain containing the corresponding target) and a negative control (distilled water).

Sequencing

A selection of *agg*R-positive pools were further tested using a cPCR with the same primers as were used for generating the standard curves. The PCR products were then sent for sequencing to Stabvida (Costa da Caparica, Portugal).

Isolation of VAG positive colonies

First, samples belonging to the pool which yielded the highest gene copy number for all VAGs of the German outbreak strain (*sxt*2, *agg*R, wzx_{O104} and $fliC_{H4}$) were analyzed individually for these VAGs using the same qPCR (see Fig. S1). To increase the chances of isolation, only the single sample in which the highest values for the mentioned VAGs were obtained was plated on Mac-Conkey agar. Thereafter, 255 individual colonies were randomly selected and tested for the presence of all VAGs.

In parallel, 26 fecal samples from six German pools positive to wzx_{O104}, $fliC_{H4}$, *agg*R and *stx*2, were further investigated for the presence of O104:H4 strains using a colony blotting technique (13). Additionally, 22 single fecal samples (non-previously tested) from the farms with positive pools were also included (Fig. S1). In brief,

a DIG-labelled rbf_{O104}DNA-probe was constructed with rbf_{O104} (forward and reverse) primers (14) using a PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer. Up to 10 colonies per sample yielding presumptive rbf_{O104} positive results in the colony blotting analysis were selected (total number of isolates = 147) for further characterization by a multiplex cPCR targeting rfb_{O104}, *fli*C_{H4}, *stx*2, *ter*D, *agg*R, *pic* and *ast*A (Table S3). Of these, 109 rfb_{O104}-PCR positive colonies were tested by seven conventional monoplex PCRs for marker genes of *p*AA plasmids (*agg*R, *agg*A, *aaf*A, *agg*3A, *agg*C, *aaf*C, *agg*3C) (14, 15). Primers were purchased from Eurofins Genomics GmbH (Ebersbach, Germany) and are listed in Tables S3 and S4. Conditions set for these PCRs are described in the Supplementary Materials and Methods section.

Statistical analysis

The proportions of samples positive to each VAG per country/slaughterhouse were compared using Fisher's exact and χ^2 tests. Quantitative results were assessed for normality and compared depending on the gene/country of origin using non-parametric tests (Mann–Whitney and Kruskal–Wallis tests). Associations between the presence of EHEC/EAEC typical VAGs and serotype markers were evaluated using the McNemar test. The level of significance was set at $P < 0.05$. All analyses were carried out using SPSS software version 20 (IBM statistics).

RESULTS

Primer specificity and efficiency of qPCRs

All the expected targets from the *E. coli* reference strains were correctly amplified in multiplex qPCRs; however, no amplification products were obtained from the negative control strains. In all cases the qPCR efficiencies were always optimal (90% to 100%) and the coefficient of determination always higher than 0.9, with a slope equal to −3.3 in most assays. All the dilutions from the *E. coli* reference strains were correctly amplified in the three replicates, and the Ct values of the three replicates were similar for all the genes. The smallest number of copies detected in the fecal samples was less than 10.

qPCR screening of pooled bovine fecal samples

Six hundred and twenty-seven bovine fecal samples were analyzed in pools (Fig. S1), each pool representing a single farm (134 pools in total: Germany, $n = 68$; Spain, $n = 66$). According to qualitative and quantitative assessment of VAGs representing the main *E. coli* pathotypes, all pools were negative for the LT encoding gene *elt*, whereas the percentages of positivity for all other VAGs ranged from high (>60%: *stx*2, *eae*, *ehx*A), to moderate (25–60%: *invA*, *stx*1, *agg*R) to low values (≤25%: *est* and *bfp*A; Table 1). Significant differences between countries ($P < 0.05$, χ^2 test) in qualitative values (i.e., proportion of positive samples) for specific genes were observed: significantly more Spanish pools were positive for *stx*1, *eae* and *inv*A, whereas significantly more German pools harbored *agg*R and wzx_{O104} in association with *fli*C_{H4}. Positive results for rbf_{O157} plus *fli*C_{H7} were significantly associated (McNemar test $P < 0.001$) with detection of STEC marker genes (*stx*2, *eae*, *ehx*A) across countries. Similarly, significant associations (McNemar test, $P < 0.001$) were observed between wzx_{O104} and *fli*C_{H4} and between *stx*2 and *agg*R. All genes characteristic of the *E. coli* O104:H4 outbreak strain (*stx*2, *agg*R, wzx_{O104} and *fli*C_{H4}) were detected simultaneously in only six pools, all of which originated from a single German abattoir (Number 4; Fig. 1).

In Spain, the number of farms sampled at each slaughterhouse ranged from one to eight, thus preventing statistical assessment of a possible abattoir-clustering factor. In Germany, where at least nine or more farms were sampled in all but one slaughterhouse (which was excluded from further calculations), significant differences were found in VAG prevalence at the farm level (Fisher exact test, $P < 0.05$) for all VAGs except *stx*2 and *est*. Nevertheless, the total number of pools positive for at least one VAG did not differ significantly between the abattoirs from which they were collected (Kruskal–Wallis test, $P = 0.92$). Pools positive for rbf_{O157} plus *fli*C_{H7} were found homogeneously across abattoirs (Fisher exact test, $P = 0.26$). In contrast, a single slaughterhouse (Number 4) situated in Northwest Germany near the outbreak epicenter, accounted for 8/11 (72.7%) wzx_{O104} plus *fli*C_{H4} positive pools, resulting in significant (Fisher exact test, $P = 0.013$) differences between slaughterhouses in the prevalence of positive pools for these targets. All six pools that were positive for the combination of *stx*2, *agg*R, wzx_{O104} and *fli*C_{H4} genes were collected in this slaughterhouse. None of the wzx_{O104} plus *fli*C_{H4} positive pools ($n = 3$) collected in other German slaughterhouses were also positive for *agg*R.

Quantitative analysis (i.e., gene copy numbers detected per mg of pooled feces) also revealed significant differences between different VAGs and country of origin (Table 1). Among VAGs for which five or more

Table 1. Qualitative and quantitative assessment of pooled bovine fecal samples for the presence of virulence associated *E. coli* genes in Spanish and German cattle herds

Target	Country	No. positive pools [%]	*P*-value†,§ (country comparison)	Mean no. of gene copies per mg feces (95% CI)
***stx*1**‡	Spain	33 (50)	0.005	1,905 (766–3043)
	Germany	18 (26.5)		326 (6.51–645)
***stx*2**‡	Spain	42 (63.6)	0.39	8,373 (0–16,941)
	Germany	48 (70.6)		3,201 (0–6715)
eae‡	Spain	62 (93.9)	0.039	1,671 (815–2526)
	Germany	56 (82.3)		106 (0–230)
***ehx*A**	Spain	65 (98.5)	0.18	240 (73.9–4079)
	Germany	64 (94.1)		131 (72.1–190)
est	Spain	4 (6.1)	0.16	236 (0–517)
	Germany	1 (1.5)		27 (NA)
***inv*A**‡	Spain	44 (66.7)	0.005	147 (59–236)
	Germany	29 (42.6)		11 (6.0–16)
***bfp*A**	Spain	6 (9.1)	0.22	3.0 (0–7.4)
	Germany	11 (16.2)		7.4 (0–17)
***agg*R**‡	Spain	8 (12.1)	<0.001	24 (13–36)
	Germany	35 (51.5)		8.4 (4.2–13)
rbf_{O157}	Spain	33 (50)	0.395	2,423 (455–4392)
	Germany	29 (42.6)		1,407 (0–2875)
$fliC_{H7}$	Spain	42 (63.6)	0.069	33,518 (1900–65,136)
	Germany	53 (78)		18,295 (7377–29,214)
wzx_{O104}	Spain	3 (4.54)	0.028	540 (0–2244)
	Germany	11 (16.2)		992 (0–2083)
$fliC_{H4}$	Spain	4 (6.1)	<0.001	3,810 (0–9930)
	Germany	24 (35.3)		6,686 (1235–12,137)
rbf_{O157}/$fliC_{H7}$	Spain	33 (50)	0.31§	NA
	Germany	28 (41.2)		
wzx_{O104}/$fliC_{H4}$	Spain	3 (4.54)	0.028§	NA
	Germany	11 (16.2)		

Number and 95% CIs of positive pooled fecal samples and mean and 95% confidence interval (CI) number of copies/mg in positive fecal pools for each molecular target.

†, Fisher's exact test/χ^2 test depending on sample size; ‡, significant differences (Mann–Whitney test, $P < 0.05$) in the quantitative results (i.e., associations between target and country);§, *P* values (χ^2test) for those positive pools with simultaneous detection of rbf_{O157} plus $fliC_{H7}$ and wzx_{O104} plus $fliC_{H4}$, respectively.

NA, not applicable.

fecal pools from both countries were positive, significantly higher numbers of copies for *stx*1, *stx*2, *eae*, *inv*A and *agg*R were detected in those from Spain (Mann−Whitney test, $P < 0.05$). Similar quantities were detected in samples from both countries for the remaining VAGs (*ehx*A, *bfp*A and *est*) as well as for rbf_{O157}, wzx_{O104}, $fliC_{H4}$ or $fliC_{H7}$.

Sequencing of the presumptive *agg*R products confirmed the specificity of the PCR: the sequence of amplicons was 100% identical to that of the 2011 EHEC/EAEC O104:H4 outbreak strain (14), as well as EAEC O104:H4 strain 55989 (16) and HUSEC 41 strain (17).

Isolation of VAG positive *E. coli* strains

Testing of 255 colonies obtained from a single bovine sample by multiplex real-time PCR (Fig. S1) suggested that VAGs were detectable using bacteriology in selected colonies only when real-time PCR values in the pool were $\geq 10e^{+03}$ gene copies per mg of feces in the pool (Table 2). Only one isolate was wzx_{O104} positive; it carried the $fliC_{H7}$ antigen without any of the other analyzed VAGs. Three isolates harbored rbf_{O157} and $fliC_{H4}$ simultaneously. Being *ehx*A the only detected VAG ($n = 11$) in the 255 colonies.

When 48 individual German fecal samples were plated, 147 colonies hybridized with a rbf_{O104}-specific probe in 21 of the samples. Of these, 109 randomly selected colonies were confirmed to be rbf_{O104} positive by multiplex PCR while being negative for $fliC_{H4}$, *stx*2, *ter*D, *agg*R, *pic* and *ast*A. No positive results with cPCRs for *agg*R, *agg*A, *aaf*A, *agg*3A, *agg*C, *aaf*C and *agg*3C, as described by Bernier and Boisen (15, 18), were obtained in these colonies.

Table 2. Positive isolates obtained from a single sample within one of the positive pools

Target	Gene copies/ mg feces ($n = 1$ pool)	Gene copies/mg feces ($n = 1$ sample)	No. positive isolates ($n = 255$ colonies tested in total)
***fliC*$_{H4}$**	4.8E + 04	2.11E + 04	65
***fliC*$_{H7}$**	4.5E + 04	NA	15
***wzx*$_{O104}$**	3.16E + 03	1.43E + 02	1
ehxA	2.2E + 01	NA	11
***rbf*$_{O157}$**	1.8E + 04	NA	3
stx2	1.75E + 02	1.44	0
invA	1.19	NA	0
eae	0.37	NA	0
aggR	0.27	1.64E + 01	0
stx1	0	NA	0
bfpA	0	NA	0
***est* (ST)**	0	NA	0
***elt* (LT)**	0	NA	0

VAGs were only detected when real-time PCR values in the pool were equal or higher than $10e^{+03}$ gene copies per milligram of feces.
NA, not analyzed.

DISCUSSION

Ruminants are well-known reservoirs of STEC strains that can be harmful for humans (and are then referred to as EHEC) (19) and have also been reported to be carriers of ETEC and atypical EPEC (20, 21). However, there is a lack of knowledge about the possible role of cattle in the epidemiology of other pathotypes. This is partially because there is a lack of sensitive and fast techniques for typing strains that are not one of the known pathotypes for which there are specific laboratory tests (22) leaving identification of a proportion of colonies arbitrarily selected from those growing from fecal samples as the only practicable approach. In our study, our combined use of a set of real-time qPCRs circumvented this obstacle and enabled a rapid first-line screening for VAGs with a very low detection limit (0.12–10 gene copies/mg of cattle feces for all targets). Use of a set of qPCRs allowed both detection and quantification of bacterial DNA, providing greater analytic sensitivity than that of cPCR (10). Because we pooled individual samples from each herd, the herd (rather than the animal) was considered the unit of analysis. In addition, analysis of pooled samples allowed us to maximize the number of cattle that could be screened and thus maximize the efficiency of detection. This straight-forward workflow detected all but one of the selected VAGs (*elt*) in the cattle samples and, moreover, it revealed significant differences as well as coincidences in the prevalences and gene copy numbers between VAGs and countries.

Using the traditional culture-based approach recommended by the EFSA, the overall prevalence of STEC in cattle (the only *E. coli* pathotype subjected to surveillance in cattle in Europe) in the European Union ranged between 2.2 and 6.8% in the 2007–2009 period, with 0.5–2.9% of STEC belonging to the O157:H7 serotype (22). Depending on the country of origin, STEC prevalence varied from 0 to 48.5% (8, 23, 24). This wide range is probably attributable to the different methodologies used; however, it may be also have been caused by an underestimation of the true prevalence of STEC that can occur when only traditional culturing methodologies and/or conventional PCR are used to monitor this pathotype. Given the overall proportion of samples testing positive for STEC-associated genes, our data support this contention. Although the simultaneous detection of *rbf*$_{O157}$, *fliC*$_{H7}$, *stx*2, *eae* and *ehx*A in the same sample does not prove the presence of strains harboring all genes at the same time, the high quantitative values found for these genes in some of the samples, coupled with the significant associations between the presence of each one with respect to the others, suggest that this may in fact be the case. Regarding VAGs belonging to other pathotypes, the very low prevalence of *est* and the absence of *elt* is in agreement with a previous study in Turkey that assessed the presence of ETEC in healthy cattle. These researchers found a low prevalence for *est* and *elt* (12.7% and 1.8% respectively) (25). The *inv*A gene was found here at a higher frequency than expected given that, although EIEC-related genes such as *ipa*H have been reported in animals, strains harboring *inv*A have been absent (26) or rare (7) in previous studies, and humans are considered the only known reservoir of EIEC (27). The same applies to the *bfp*A gene (28, 29), which we found in both German and Spanish fecal samples (and confirmed it in a number of Spanish isolates) at different frequencies. We also detected low copy numbers of genes indicative of typical EPEC strains ($eae^{+}bfpA^{+}$) in the cattle samples, in agreement with other authors who postulated that there are other reservoirs for EPEC in addition to the human one (19).

Thus far, no animal reservoir had been identified for EAEC, although in a previous study *E. coli* VAGs of all human pathotypes, including some EAEC-related genes (*agg*R among them), were detected in fecal samples from cattle, chickens and pigs from Burkina Fasso (25). In this study, PCR amplicon sequences generated from samples collected in the present study matched 100% the *agg*R sequence of the German outbreak and other O104:H4 strains but were less similar (<99%) to the *agg*R of other EAEC strains such as *E. coli* 042 or *E. coli* plasmid p86A1 (13). A recent study conducted at one of the institutions participating in this study (Friedrich—Loeffler-Institut)

showed that the *E. coli* O104:H4 outbreak strain can colonize calves upon experimental infection similar to an O157:H7 strain (Hamm, Menge *et al.*, pers. comm., EXPERIMENTAL INFECTION OF CALVES WITH ESCHERICHIA COLI O104:H4 K. Hamm, S. Barth, S. Stalb, E. Liebler-Tenorio, J.P. Teifke, E. Lange, G. Kotterba, E. Dean-Nystrom, M. Bielaszewska, H. Karch, C. Menge, Germany . German Symposium on Zoonoses Research 2014 and 7th International Conference on Emerging Zoonoses. October 16, 2014, Berlin, Germany.). Detection of *agg*R in cattle fecal samples further indicates that at least some EAEC-type strains are able to circulate in bovine hosts, implying that the paradigm that humans are the only EAEC reservoir needs to be revised.

The analysis of samples from two countries allowed us to detect differences in the distribution of certain VAGs in cattle from several areas. We found that six (8.8%) of the German cattle herd samples were simultaneously positive for *stx*2, *agg*R, wzx_{O104} and $fliC_{H4}$, the most important genetic markers of the *E. coli* O104:H4 outbreak strain (14). In contrast, no Spanish samples were simultaneously positive for the combination of these four markers, in agreement with what has been reported for French cattle (30). Additionally, while we detected *agg*R alone in eight (12.1%) of the Spanish samples, we detected it in a higher proportion among the German samples (35/68 samples, 51.5%). Interestingly, all German *agg*R positive samples were collected in only two abattoirs (four and six) that were located close to each other (Fig. 1). All samples positive for *stx*2, *agg*R, wzx_{O104} and $fliC_{H4}$ simultaneously came from a single abattoir located closer to and, even more importantly, processing animals from farms located closer to, the HUS outbreak epicenter. Although we did not isolate the outbreak strain here, this is in agreement with a previous study performed when the outbreak was still ongoing, in which typing of selected colonies from 100 fecal samples also failed to yield any O104:H4 isolates (9). In addition, the EHEC/EAEC O104:H4 strain was not recovered from the suspect fenugreek seeds when these were plated on specific media (31). In 2011, EFSA stated that this failure was not totally unexpected because of the possibly low levels of contamination. Furthermore, some *E. coli* strains, including the outbreak strain, may enter into viable but non-culturable (VBNC) states under stress conditions but, when conditions for its growth are favorable, these strains can grow and induce expression of virulence factors (32). Thus, after the German outbreak, the STEC Workshop Reporting Group recommended implementation of novel methodologies for detecting VBNC *E. coli* strains as well as the development of new molecular surveillance techniques (33). The fact that positive colonies for a particular VAG were only recovered from individual fecal samples when at least 10^3 gene copies/mg feces were present in the sample pool could explain neither *agg*R positive colonies nor the outbreak strain were isolated in this study and in the preceding studies. Even if these genes were not present within a single *E. coli* strain, their simultaneous occurrence in a certain epidemiological setting (such as a herd) represents a potential risk given their mobility, as it could give rise to the emergence of pathogenic strains. The analytic workflow used here has the potential to unveil such scenarios and could therefore be useful for risk-assessment.

Additionally, the 1–1.5 year interval that elapsed between the outbreak and the sampling carried out in this study may have allowed local spread of the bacteria in the human population and spill-over into the environment, including livestock. The finding that a significantly higher proportion of German samples were positive simultaneously for the four genetic markers of the *E. coli* O104:H4 German outbreak strain (*stx*2, *agg*R, wzx_{O104} and $fliC_{H4}$) may indicate that the introduction of a highly human-pathogenic *E. coli* strain to a geographic area may not only affect human health (3), but also can introduce new VAGs into the local cattle *E. coli* population.

In conclusion, direct detection and quantification of *E. coli* VAGs in cattle samples revealed higher prevalence than expected at the herd level for most of the selected VAGs belonging to all human pathotypes (and including those characteristic of the *E. coli* O104:H4 outbreak strain). Moreover, we found significant differences depending on the VAG and country of origin of the sample. The use of pooled samples allowed the identification of risk profiles at the herd level in a rapid and cost-efficient manner that could be useful for detection of high risk situations before outbreaks occur.

ACKNOWLEDGMENTS

The authors wish to thank our technicians M.C. Comeron, N. Maasoumi, L. del Moral (Spain) and A. Hinsching (Germany) for their excellent work. We also thank Dr. Martina Bielaszewska, Institute of Hygiene and the National Consulting Laboratory for Hemolytic Uremic Syndrome, University of Münster, Germany for providing some *E. coli* reference strains. This study was funded by EU FP7 Antigone (Project Number 278976). A. Cabal was a recipient of a pre-doctoral grant for internships in foreign research institutes (Cátedra Enresa−UCLM).

DISCLOSURE

All authors declare they do not have any conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Text S1. Supplemental materials and methods. PCR conditions set for the outbreak strain marker genes. PCR conditions set for the *p*AA marker genes.
Fig. S1. Analytic workflow for processing of samples and isolates. The diagram depicts the numbers of sampled feces, the techniques consecutively applied and the results obtained in each step. Based on first-line findings, the overall strategy was amended according to the country of origin of the samples. cPCR, conventional PCR.
Table S1. qPCR probes developed and used.
Table S2. Primers developed and used for standard curves.
Table S3. Target genes and oligo sequences for multiplex PCR.
Table S4. Primer sequences for detection and differentiation of pAA plasmids.