Virulence gene profiles of rabbit enteropathogenic Escherichia coli *strains isolated in Northern Italy*

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Summary
The virulence gene profile of 26 rabbit enteropathogenic *Escherichia coli* strains, isolated from 17 colibacillosis outbreaks located in two regions of Northern Italy, was determined using an *Echerichia coli* virulence DNA microarray. All strains were classified according to their determined biotype, sero- and phylo-group. The distribution of virulence genes encoding for the Locus of enterocyte effacement (LEE), LEE type III secretion system (T3SS), non-LEE T3SS translocated proteins and adherence factors was also determined. All strains but one belonged to phylogroups A and B1. A prevalent association between the O103 serogroup with the rhamnose-negative phenotype (biotype 12 or 14) was found. The most prevalent LEE profile found in tested strains was *ler/cesT/espA-1/espB-3/tir-1/eae(beta)/espD-2/escN/eprJ*. All strains possessed either the adhesive factor rabbit-2 (*afr/2*) or the plasmid Rabbit adherence locus (*ral*) gene and 24 of them an additional individual or combined set of colonization factors *efa1/lifA, lpfA* and *paa* genes. Finally, the combined or single presence of a set of LEE and/or non-LEE effector proteins encoding genes, namely *espG, cif, map* and *nle* family genes, attested to the genetic potential of investigated strains to induce pathologic lesions to the host. The application of microarray-based technologies in assessing the genetic profile of rabbit *E. coli* is a reliable, cost-effective candidate for large scale investigations in monitoring programs aimed to survey the circulation of pathogenic strains within rabbit production units, their zoonotic genetic potential and to select *E. coli* strains eligible for vaccinal prophylaxis in fattening rabbit production.

Profilo dei geni di virulenza di ceppi enteropatogeni di Escherichia coli *isolati in conigli del Nord Italia*

Parole chiave
Colibacillosi,
Coniglio,
DNA Microarray,
EPEC atipico,
Escherichia coli,
REPEC.

Riassunto
Nel presente lavoro è stato determinato il profilo dei geni di virulenza di 26 ceppi enteropatogeni di *Escherichia coli* con la tecnica del DNA microarray; i campioni sono stati prelevati da 17 focolai di colibacillosi rilevati in conigli provenienti da due regioni del Nord Italia. I ceppi sono stati classificati secondo il loro biotipo, il sierogruppo e il gruppo filogenetico. È stata anche precisata la distribuzione dei geni di virulenza che codificano il Locus di cancellazione degli enterociti (LEE), il quello del sistema di secrezione tipo 3 (T3SS), le proteine non-LEE traslocate tramite il T3SS e i fattori di adesione. I ceppi testati, ad eccezione di 1, appartengono ai gruppi filogenetici A e B1. È stata osservata, inoltre, un'associazione predominante tra il sierogruppo O103 e il fenotipo ramnosio-negativo (biotipi 12 o 14). Il profilo del LEE maggiormente riscontrato è stato *ler/cesT/espA-1/espB-3/tir-1/eae(beta)/espD-2/escN/eprJ*. Tutti i ceppi possedevano il fattore di adesione rabbit-2 (*afr/2*) o il *ral* (Rabbit Adherence Locus) e 24 di essi un ulteriore set costituito da uno o più tra i fattori di colonizzazione *efa1/lifA, lpfA* e *paa*. Infine, la presenza singola o combinata di proteine effettrici LEE e/o non-LEE, che codificano i geni *espG, cif, map* e *nle*, ha attestato la potenzialità genetica che hanno i ceppi studiati ad indurre lesioni patologiche all'ospite. Le tecnologie basate sul microarray, applicate alla valutazione del profilo genetico dell'*Escherichia coli* del coniglio, si sono dimostrate convenienti e affidabili se finalizzate ad indagini a larga scala all'interno di programmi di sorveglianza per monitorare la circolazione

di ceppi patogeni nella filiera di produzione del coniglio, valutare il loro potenziale genetico zoonotico, e selezionare ceppi patogeni di *E. coli* per creare una profilassi vaccinale mirata nelle unità da ingrasso degli allevamenti cunicoli.

Introduction

Based on their virulence gene content and overt clinical symptoms, *Escherichia coli* strains are classified into different pathotypes responsible for intestinal or extra-intestinal syndromes. In particular, the attachement and effacing *E. coli* (AEEC) pathotype, which includes both enteropathogenic (EPEC) and enterohemorrhagic (EHEC) strains, are characterized by the presence of a chromosomal pathogenicity island called the Locus of enterocyte effacement (LEE), encoding for proteins responsible for the attaching and effacing (A/E) phenotype. AEEC strains that are classified as EHEC also possess the Shiga toxin encoding-genes *stx1* and/or *stx2*. LEE genes are organized in five operons, named LEE1 to 5. LEE1, LEE2 and LEE3 transcribe the Type III Secretion System (T3SS) *E. coli* secretion (Esc) proteins and LEE4 transcribes the *E. coli* secreted proteins (Esp). LEE5 operon contains *eae*, the gene encoding the bacterial adhesin Intimin which is involved in the intimate attachment to host epithelial cells, its receptor *tir* (translocated intimin receptor) and the chaperone *cesT*, translocated from the bacteria to the host epithelial cells through the T3SS structure. The T3SS is also used to inject LEE and non-LEE encoded effector proteins (EspCGP, Map; Cif, Nle) into the host cell, which are responsible for the effacement of host epithelial cells (Zhu *et al.* 2001, Marchés *et al.* 2003, Schmidt and Hensel 2004, Bertin *et al.* 2004, Thomas *et al.* 2005, Garrido *et al.* 2006, Luo and Donnenberg 2011).

EPEC strains also possess the *E. coli* adherence factor (EAF) virulence plasmid, that encodes the adhesin Bfp (bundle forming pili), which induces localized adhesion (LA) of bacterial microcolonies on epithelial cells. The LA phenotype is associated with serotypes/serogroups from human diarrhea outbreaks (Giron *et al.* 1991). EPEC strains, which lack the EAF plasmid, are called atypical EPEC (aEPEC) (Nuyen *et al.* 2006).

After the initial study by Cantey and Blake (Cantey and Blake 1977) on the rabbit diarrheal *E. coli*-1 strain (RDEC-1), strains involved in rabbit colibacillosis outbreaks in several European countries were confirmed as non-toxin producing aEPECs inducing A/E lesions (Milon *et al.* 1999). The pathogenic specificity of rabbit EPEC (REPEC) strains lies primarily with the adherence mechanism that complements attachment to host epithelial cells, mediated by Intimin. Afr-2 (adhesive factor rabbit-2), a chromosomal encoded fimbrial adhesin inducing the phenotype of 'diffuse adhesion' (DA) on surface of HeLa cells, is associated with virulent rabbit strains (Fiederling *et al.* 1997). In addition, a putative chromosomal pathogenicity islet gene Porcine A/E-associated (Paa), which strongly correlates with the A/E phenotype, a chromosomal Long polar fimbriae gene (Lpf), thought to contribute to epithelial cell colonization, and a plasmid-encoded fimbriae Repec adherence locus gene (Ral), involved in the early steps of adhesion, were also found in diarrheal rabbit *E. coli* isolates (Batisson *et al.* 2003, Badea *et al.* 2003, Newton *et al.* 2004, Krejany *et al.* 2000).

The *eae* and *afr/2* genes are the main genetic markers used to define REPEC strains (Milon *et al.* 1999). Another way to determine *E. coli* enteropathogenicity is to assess both the serogroup and sugar fermentation character (biotype), targeted to establishing a link between biotype/serotype and high mortalities (Camguilhem and Milon 1989).

An oligonucleotide virulence microarray, initially developed and validated as previously described (Bekal *et al.* 2003, Bruant *et al.* 2006), allowed the detection of an exhaustive list of *E. coli* virulence genes as well as antimicrobial resistance genes in *E. coli* strains isolated from coastal water and wastewater (Hamelin *et al.* 2006, Frigon *et al.* 2013), camel (Salehi *et al.* 2012), poultry (Bonnet *et al.* 2009), and cattle (Staji *et al.* 2017). In a previous study this microarray was successful in clustering a group of aEPEC strains from rabbit experiencing diarrhea from a second cluster that grouped non-pathogenic *E. coli* strains from healthy rabbit, both coming from the same rabbitry (Tonelli *et al.* 2008).

The aim of the present work was to develop a methodology for defining virulent genotypes of REPEC strains by genotyping a collection of rabbit enteropathogenic *E. coli* strains isolated from rabbitries affected by colibacillosis in Northern Italy.

Materials and methods

Strain collection, microbiology

Twenty six *E. coli* extracted DNA, tested for *eae* polymerase chain reaction (PCR) signal carried out as previously described (Agnoletti *et al.* 2004),

were selected from a more extensive collection coming from strains isolated from rabbits affected by diarrheal syndrome. Rabbits were 7- to 87-day old coming from 17 rabbitries located in the Veneto and Piemonte regions (Northern Italy) with variable pathological degree of lesion ranging from liquid content in the caecum to typhlitis, enterotyphlitis and severe enterotyphlitis. An inoculum from the caecal contents of diseased animals was directly plated onto Eosin methylene blue agar (EMB, Oxoid Ltd, England) and *E. coli* isolates identified using the API 20E® system (BioMérieux, France) and biotyped according to the method described by Camguilhem and Milon (Camguilhem and Milon 1989). One colony was re-isolated on tryptic soy agar (Biolife Italiana, Milan, Italy), suspended in phosphate buffer saline and its DNA extracted with a GenElute Bacterial Genomic DNA kit® (Sigma-Aldrich, USA) and stored at -20 °C until use.

Microarray design and *E. coli* virulence genes or markers

The microarray version used in the present study was composed of 70-mer oligonucleotide probes printed in duplicate on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario), targeting for 348 *E. coli* virulence factors, covering all known *E. coli* pathotypes, and 95 antimicrobial resistance genes. The tryptophanase (*tnaA*), beta-glucuronidase (*uidA*), lactose permease (*lacY*) and beta-galactosidase (*lacZ*) genes were included as positive controls whilst negative controls included empty buffer spots as well as genes for the green fluorescent protein of *Aequoria victoria* (*gfp*) and the chlorophyll synthase gene of *Arabidopsis thaliana* (*At3g*). Only data related to virulence or virulence-related gene hybridizations were considered in this study, since the antimicrobial resistance profiles of the same strains were analyzed separately (Badagliacca *et al.* 2014). Confirmatory tests independent of the microarray, were performed by specific molecular probes or gene sequencing, as described below.

Escherichia coli isolates were assigned to a serogroup based on their *wzy* gene for O7, O15, O22, O24, O26, O28, O45, O53, O55, O56, O59, O66, O86, O91, O98, O103, O104, O113, O114, O117, O121, O123, O126, O127, O128, O138, O139, O141, O145, O146, O147, O148, O149, O155, O157, O172, O174, and O177. Assignment to other serogroups was based on rfc gene for O4, *wzx* for O6, *wb* for O8, *rfb* for O9 and O101 and *wbdI* for O111.

E. coli phylogenetic groups were identified on the microarray based on the combined presence/absence of the *chuA* gene, the *yjaA* gene and the DNA fragment TSPE4.C2, according to Clermont and colleagues (Clermont *et al.* 2000).

E. coli isolates were clustered based on their biotype/serogroup/phylogroup profile and analysed according to the virulence gene or marker patterns related to LEE structural genes (*ler, eae, tir1-2-3, cesT, espABD, escJN, eprJ*), LEE and non-LEE effector (*espCGP, map/orf19; nleABCDEFGH, cif*) and adherence/colonization factors (*afr/1-2, ralG, bfpAB, paa, efa1/lifA, lpfA*). Finally, LEE gene variant combinations were classified according to Afset *et al.* (2008). The chi-square test was used to assess the associations between serogroup, biotype, and phylogroup. Statistical significance was expressed with *p*-value ($\alpha < 0.05$).

E. coli DNA labeling and hybridization

Approximately 300 ng of purified genomic DNA was labelled with fluorescent Cyanine (Cy3) dye using the Bioprime DNA labelling system (Invitrogen Life Technologies, Burlington, ON, Canada) as described previously (Bruant *et al.*, 2006). Labelling efficiency and the percentage of dye incorporation were then determined by scanning the DNA sample in a NanoDrop spectrophotometer from 200 to 700 nm. Cy3 dye incorporation was calculated using a Web-based percent incorporation calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html). Pre-hybridizations and hybridizations were performed following a protocol derived from Hamelin and colleagues (Hamelin *et al.* 2006). For the hybridizations, 500 ng of labelled DNA was dried under vacuum in a rotary desiccator without heating (Savant SpeedVac®, ArrayIt, USA). Dried labelled DNA was resuspended in hybridization buffer (DIG Easy Hyb Buffer, Roche Diagnostics, Laval, Quebec, Canada). Microarrays were pre-hybridized for one hour at 50 °C with a pre-heated pre-hybridization buffer containing 5X SSC, 0.1% SDS and 1.0% BSA. After pre-hybridization, the microarrays were hybridized with a solution that consisted of 25 µl of hybridization buffer, 20 µl of Bakers Yeast tRNA (10 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) and 20 µl of Sonicated Salmon Sperm DNA (10 mg/ml) (Sigma Aldrich, St. Louis, MO, USA), mixed together with the labelled DNA which had previously been denatured. Microarrays were hybridized overnight at 50 °C in a SlideBooster (model SB800; Advalytix, Germany). After hybridization, stringency washes were performed with Advawash (Advalytix, Germany) using 1X SSC, 0.02% SDS preheated to 50 °C.

Microarray data acquisition

Microarray slides were scanned with a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Missasauga, Ontario, Canada) using with ScanArray Gx software (Perkin-Elmer, Foster City, CA). Fluorescent spot intensities were quantified

with QuantArray Version 3.0 (Packard Bioscience, Informer Technologies Inc., USA).

The mean value for each set of duplicate spotted oligonucleotides was divided by the correction factor taken from the negative control spots. This value was then divided by the average of the empty spots to create a signal-to-noise ratio. Oligonucleotide spots with a signal-to-noise fluorescence ratio greater than the established threshold (2 in this study) were considered positive. These ratios were then converted into binary data where a value of 0 indicates a negative probe and a value of 1 indicates a positive probe.

Confirmatory and additional tests

Strains showing LEE structural genes demonstrating a signal lower than the threshold were confirmed by PCR. A confirmatory PCR test for *espB* was designed with Primer-BLAST™, after a gene sequence alignment of 2,267 blast hits present in GenBank. Two primers, forward 5'-GCCGTTTTTGAGAGCCA-3' and reverse 5-TTACCCAGCTAAGCGA-3', were synthesized (Eurofins MWG Operon, Ebersberg, Germany). Confirmatory test of *tir* gene as well additional PCRs for variants 1, 2 and 3 of *espD*, not hybridized by microarray, were performed as described by Garrido and colleagues (Garrido *et al.* 2006). Additional PCR for the *cesT* gene was performed using primers from Bertin and colleagues (Bertin *et al.* 2004). All PCRs were carried out using universal master mix (PCR Master Mix 2xTM, Promega Italia srl, Milan, Italy), according the provider's instructions.

Strains showing an ambiguous signal for REPEC fimbrial factors Afr or Ral were submitted to genomic sequencing. DNA samples were quantified by using the Qubit® DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). One ng of DNA was used for library preparation by using the Nextera XT Library Prep kit (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Deep Sequencing was performed on the NextSeq 500 (Illumina Inc., San Diego, CA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles and standard 151 bp pairs-end reads.

Table I. *Strain identification, pathological/epidemiological data, biotype and genetic grouping of enteropathogenic* Escherichia coli *strains collected from rabbitries of Northern Italy.*

Strain*	Age (day)	Unit	Pathological lesion	Biotype	O-type#	Phylogroup	Profile°
VR/A-1	65	Fattening	Enterotyphlitis	B30	NT	B1	II
VR/A-2	65	Fattening	Enterotyphlitis	B14	O145	A	III
TV/B-1	70	Fattening	Typhlitis	B30	O103	A	I
TV/B-2	70	Fattening	Typhlitis	B14	NT	A	I
TO/C-1	56	Fattening	Enterotyphlitis	B12	O103	B1	I
TO/C-2	42	Weaning	Liquid content in caecum	B14	O103	B1	I
TO/C-3	43	Weaning	Liquid content in caecum	B12	O103	B1	I
TV/D-1	60	Fattening	Enterotyphlitis	B12	O148	A	I
TV/E-1	68	Fattening	Enterotyphlitis	B12	O103	B1	I
PD/F-1	50	Weaning	Enterotyphlitis	B12	O157	B1	I
VE/G-1	50	Weaning	Severe enterotyphlitis	B30	O145	B1	II
TV/H-1	25	Nest	Severe enterotyphlitis	B30	O103	B1	I
TV/I-1	21	Nest	Enterotyphlitis	B12	O103	B1	I
TV/I-2	42	Weaning	Enterotyphlitis	B12	O103	D	I
TV/L-1	55	Fattening	Enterotyphlitis	B14	NT	B1	I
PD/M-1	7	Nest	Enterotyphlitis	B28	O145	B1	IV
PD/M-2	48	Weaning	Enterotyphlitis	B30	O15	B1	II
TV/N-1	48	Weaning	Enterotyphlitis	B12	O103	B1	I
TV/O-1	75	Fattening	Enterotyphlitis	B12	O126	B1	I
TV/L-2	70	Females	Enterotyphlitis	B14	O103	B1	I
TV/L-3	42	Weaning	Severe enterotyphlitis	B12	NT	B1	I
TV/L-4	43	Weaning	Severe enterotyphlitis	B14	O59	B1	I
TV/P-1	87	Fattening	Enterotyphlitis	B12	O157	B1	I
TV/P-2	87	Fattening	Severe enterotyphlitis	B14	O103	B1	I
TV/Q-1	50	Weaning	Severe enterotyphlitis	B14	O103	B1	I
TV/R-1	77	Fattening	Liquid content in caecum	B28	O177	A	I

*Strain identification was defined by Province code/alphabetical code of farm-number of strain; #NT, notypeable; °See Table II.

Table II. *Profiles of Locus of Enterocyte and Effacement (LEE) and adherence factor encoding genes in rabbit enteropathogenic* Escherichia coli.

Profile	N° of involved strains	LEE structural or secreted proteins*	Fimbrial adhesin#	Adherence factors (n)	Phylogenetic group (n)	Afset group
I	21	*ler, cesT, espA-1, espB-3, tir-1, eae(beta), espD-2; eprJ, escN*	*afr/2G*	*efa1/lifA(12), paa(17), lpfA(19)*	*A (4); B1 (16); D (1)*	*D*
II	3	*ler, cesT, espA-1, espB-3, tir-1, eae(beta), espD-2; eprJ, escN,*	*ralG*	*efa1/lifA(3), paa(1), lpfA(2)*	*B1*	*D*
III	1	*ler, cesT, espA-1, espB-3, tir-3, eae(beta), espD-2; eprJ, escN*	*ralG*	-	*A*	*This study*
IV	1	*ler, cesT, espA-1, espB-2, tir-1, eae(beta), espD-2; eprJ, escN*	*ralG*	*efa1/lifA, paa, lpfA*	*B1*	*This study*

* Strains TV/H1, TV/I2 and TV/N1 possess additional *escJ* gene; *espD* and *cesT* genes were detected by specific PCRs; signal for *espB-3* variant was below the threshold signal in strains TV/B2, TO/C3, TO/D1, TV/L4, TV/P1, TV/R1, VR/A2, VE/G1 and PD/M2, confirmed by specific *espB* PCR; signal for the *tir-1* variant was below the threshold signal in strains TV/B2 and VR/A2, confirmed by specific *tir* PCR;

\# Strains TV/L-1 and TV/O-1 showing ambiguous signal for REPEC adhesins were analysed by genomic sequencing. Both strains showed 100% similarity with the described sequence of *afr/2G* (accession number U77302), 72% similarity with the sequence of *ralG* (accession number U84144). No sequence similar to *bfpA* (accession number U27184) was found.

The reads obtained were trimmed using a in-house script that trims the first 15 nucleotides and uses as cutoff a mean quality of 30 and the minimum length of 50 bases. Trimmed reads were *de novo* assembled using SPADES version 3.0.0 (Bankevich *et al.* 2012). Ambiguous nucleotides were solved by local reads remapping by Bowtie 2 (Langmead and Salzberg 2012), followed by alignment visual inspection.

Results

Epidemiological data and strain classification

Table I summarizes the epidemiological data of colibacillosis outbreaks along with the related biochemical or genetic markers used to group the *E. coli* isolates. No apparent associations were found between epidemiological data (age, production unit, lesion degree) and either the biotype or phylogroup. All but four nontypable strains possessed the O-serogroup gene *wzy*. All strains but one belonged to phylogroups A and B1 with one strain belonging to phylogroup D. B1 was the prevalent phylogroup (76.9%) and was found mostly associated with the O103, O157, O126 *wzy* gene as well as notypable ones. An association between the O103 *wzy* gene and the TSPE4.C2 DNA fragment (phylogroup B1) with the rhamnose-negative fermentation character (biotype 12 or 14) was found but wasn't statistically significant ($\alpha > 0.05$).

Pathotyping and virulence gene profiles

Table II shows the virulence-related gene profiles of *E. coli* strains and their clustering into four groups based on the presence of the rabbit-specific adherence factor encoding genes (*afr/2G* or *ralG*) and variants of the LEE structural genes. Twenty-four strains possessing the *ler/cesT/espA-1/espB-3/tir-1/eae(beta)/espD-2/eprJ/escN* LEE gene variants were classified as the Afset group D and clustered into two profile differing for *afr/2G* or *ralG* fimbrial encoding gene, respectively. Two *ralG*-positive strains differ from this latter group just for the variant *tir-3* or *espB-2*. The combined presence of adherence factors, porcine A/E-associated islet, lymphostatin and long polar fimbriae encoding genes (*paa, efa1/lifA, lpfA*) was found in 13 strains with an additional 11 strains showing a positive hybridization for at least one of the three genes.

Table III. *Frequence of effector enteropathogenic virulence factors in rabbit* Escherichia coli.

Gene	N° of isolates tested positive
LEE T3SS-effector	
espG	24
map/orf19	24
Non-LEE T3SS-secreted or structural protein	
cif	24
nleA	20
nleB	13
nleC	26
nleD	7
nleE	22
nleF	3
nleG	21
nleH	21

Table III lists the percentage of isolates possessing virulence genes related to LEE T3SS-translocated or non-LEE T3SS-secreted proteins. The *EspG* protein encoding gene was diffusely detected in the strain collection as well as effector genes *map* and *cif*. All strains possessed the non-LEE effector C, (*nleC*), together with a variable presence of *nleA, nleB, nleD, nleE, nleF, nleG* and *nleH*.

Discussion

The *E. coli* virulence DNA microarray technology has proven to be a powerful tool for large scale genetic characterization of rabbit enteropathogenic *E. coli* and has allowed us to obtain genetic profiles consistent with its pathogenesis. In particular, it has produced a database for more specific analysis of gene products. The strains examined in this study were classified as aEPEC, thus confirming that the rabbit colibacillosis agents belong to this pathotype (Milon *et al.* 1999, Licois and Marlier 2008). These strains possessed the rabbit specific adherence factors *afr/2G* or *ralG*, confirming the species-specificity of rabbit colibacillosis agents (REPEC). Interestingly, the *afr/2G* and *ralG* genes weren't detected together in our strains, suggesting that an alternative genotype is expressed by operons of REPEC adherence locus. Although ral and *afr/2* genes were not two-way associated with serogroup or biotype, the O145 (3 strains) and O103 (12 strains) serogroups were ral and *afr/2* positive, respectively.

The classification of strains according to biotype, phylogroup and O-antigen encoding gene, agreed with previous clinical and epidemiological studies on rabbit enteropathies. Indeed, all strains, except strain TV/I-2, belonged to phylogroups B1 and A, which generally encompasses *E. coli* strains derived from animal origin (Clermont *et al.* 2000). The most prevalent genetic pattern related to phylogroup/O-antigen/biotype, found in nine strains (about 35% of examined strains), was B1/O103/rhamnose negative. This is consistent with previous investigations on rabbit colibacillosis in Italy (Badagliacca *et al.* 2010, Agnoletti *et al.* 2004).

A LEE-encoding set of genes covering the *cesT* chaperon and the five operons related to the LEE regulator (*ler*) gene, the *eae/tir* domain (*eae*, *tir*) and the LEE type III secretion system (*esp, esc* and *epr*), was detected in all strains. In particular, the most prevalent combination of LEE gene variants *espA-1/espB-3/tir-1/eae(beta)* corresponds to the Afset D group. Afset and colleagues (Afset *et al.* 2008) classified atypical EPEC associated with diarrhea in children into 11 group (A to K group) on the basis of variant distribution of these four LEE genes, compared to the phylogenetic group and the presence of the adherence factor *efa1/lifA*. The Afset D group is associated with prevalent A and B1 phylogroup and *efa1/lifA* gene. Therefore, the presence of the *efa1/lifA* gene in 16 strains (61.5%) and the above referred phylogrouping results strongly correlated with this classification. Conversely, the profiles *espA-1/espB-3/tir-3/eae(beta) and espA-1/espB-2/tir-1/eae(beta)*, found in the *ralG*-harbouring VR/A2 and PD/M1 strains were not included in the Afset classification. Finally, the *lpfA* and *paa* genes were detected in 22 (84.6%) and 19 strains (73.1%), respectively. These gene products increase the colonization capability and severity of epithelial damage (Newton *et al.* 2004, Batisson *et al.* 2003).

All strains possessed the *eprJ* and *escN* genes, responsible for secretion of T3SS basal body rod component and general T3SS translocation activity, respectively, both of which are involved in the activation of the inflammation mechanism (Miao *et al.* 2010). Moreover, the confirmatory PCR test for *espB* gene and additional test for *espD* gene, together with general positive hybridizations for *espA*, attests to the ability of examined strains to carry out pore formation allowing the translocation of effector proteins. A LEE pattern, namely *espG*, *map*, as well as non-LEE (*cif, nle* family gene) effector genes were individually or in combination detected in all strains, confirming the genetic basis underlying the examined strain's ability to induce epithelial damage and an irreversible cytopathic effect.

Although no strain in our study possessed a member of the Shiga toxin gene family, finding *chuA* gene (a marker for heme transport in EHEC O157:H7) in TV/I-2 O103 strain as well as five other strains being characterized as O157 or O145 serogroup, is a reminder of the known plasticity of *E. coli*. Even in light of the recent haemolytic uraemic syndrome epidemics in Germany and France caused by bacteriophage-mediated acquisition of the *stx2a* gene by the enteroaggregative *E. coli* O104:H4 strain (Scheutz *et al.* 2011), the use of the DNA microarray technique in screening studies could be a support decision tool for more specific genetic investigations about the recombination capacity of *E. coli* pathotypes.

Conclusion

Despite the low overall number of strains tested, this study confirms that in Northern Italy rabbit colibacillosis was caused by agents having a defined genotype that was consistently found within a larger and variable virulence gene background. The rabbit species-specific adherence virulence factors, *afr/2* or *ral*, characterize the REPEC strains. Overall, a prevalent attaching and effacing gene profile consisting of *espA-1/espB-3/tir-1/eae(beta)* places the examined REPEC strains into the Afset D group of atypical EPEC. The diffuse detection of additional adherence factors as *efa1/lifA* support this classification. The link between O145 serogroup and the Ral adherence factor suggests that routine REPEC diagnosis should incorporate ral, together with *eae* and *afr/2*, as PCR amplification targets. Finally, the absence of Shiga toxin encoding genes limits the zoonotic potential of tested strains.

The application of microarray-based technologies

in the diagnosis and genotyping of pathogenic rabbit *E. coli* is a powerful tool to characterize both virulence potential and genomic lineages as well as to understand bacterial pathogenesis. It is a reliable, cost-effective candidate for more extensive investigations in monitoring programs aimed to survey the circulation of pathogenic strains within rabbit production units, their zoonotic genetic potential and to select *E. coli* strains eligible for vaccinal prophylaxis in fattening rabbit production.

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