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Molecular characteristics of *Escherichia coli* serogroup O78 strains isolated from diarrheal cases in bovines urge further investigations on their zoonotic potential

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We investigated the virulence properties and clonal relationship of 21 *Escherichia coli* strains of serogroup O78 isolated from diarrhoeic cattle and calves. Isolates were screened for 18 genes representing virulence features of different *Escherichia coli* pathotypes. None of the strains harboured enterotoxin-genes *estIa/Ib*, *eltIa*/*Ib,* or Shiga toxin (*stx*) genes, genes involved in adhesion (*eae*, *f5*, *f41*) hemolysin gene *hlyA* or invasion gene *ipaC*. With a high prevalence we detected enterotoxin *astA* (61.9%), genes involved in iron acquisition, like *fyuA*, *irp* (each 57.1%) and *iucD* (81.0%), and the operon sequence of Colicin V plasmids (38.1%). Some strains possessed toxin genes *cdt-IIIB* and *cnf1/2* (both 14.3%), the invasion gene *tia* (23.8%), and the serine protease encoding gene *espP* (23.8%). Moreover, we could show that *E. coli* O78 strains under investigation were able to adhere to and invade MDBK-cells with varying efficiencies. The results indicate that the closely related O78 strains, constituting two major PFGE-clusters, harbor various virulence features for bovine intestinal disease but cannot be grouped into one of the common *E. coli* intestinal pathogenic or other pathotypes according to their virulence gene pattern. Nevertheless, the ability to adhere, invade or harbor toxin genes lets us suggest that O78 strains isolated from diarrheal cases in bovines urges further investigations on the zoonotic potential of these strains.

Keywords: Diarrhea / *Escherichia coli* / Serogroup O78 / Zooantroponosis

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1 Introduction

Escherichia (E.) coli pathotypes associated with bovine intestinal diseases are predominantly enterotoxigenic (ETEC) and attaching and effacing (AEEC) *E. coli* [1–4]. Recently, also necrotoxigenic *E. coli* (NTEC) gained more attention in diarrheagenic infections in cattle [5, 6]. In humans, however, during the last decade, other *E. coli* pathotypes associated with diarrhea have been increasingly identified, namely enteroinvasive (EIEC), diffuse adhering (DAEC), enteroaggregative (EAEC), and certain necrotoxigenic (NTEC) *E. coli*. *E. coli* typing depends on the identification of O (lipopolysaccharide) and H (flagella) antigens, defining serotypes. Serogroup O78 strains are found in several *E. coli* pathotypes, causing numerous extra- and intraintestinal clinical symptoms in various hosts. This is why O78 strains have been the focus of several investigations [7–11]. While ETEC isolated from humans often belong to serogroup O78, in animals *E. coli* strains of serogroup O78 are commonly associated with extraintestinal disease, predominantly septicaemia [10, 12]. They are one of the predominant serogroups in avian pathogenic *E. coli* [13, 14]. During the last decade, however, diagnostic laboratories at the veterinary faculties in Giessen and Berlin, Germany, both recognized an increasing isolation of *E. coli* O78 strains from intestinal diseases in bovines. To our knowledge, it is currently unknown, whether strains of this serogroup represent a new bovine intestinal pathotype.

The objective of this study was thus to investigate bovine *E. coli* O78 strains isolated from diarrheal cases with special emphasis to their adhesive, toxigenic, and invasive properties as well as to their clonal relatedness. According

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Abbreviations: EAEC, enteroaggregative; **EHEC**, enterohemorrhagic; **EIEC**, entreoinvasive; **EPEC**, enteropathogenic; **ETEC**, enterotoxigenic *E. coli*

Table 1. *Escherichia coli* and *Shigella* reference and control strains

Strain	Serovar	Virulence factors/genes	Origin	Ref.
E. coli				
TTP-1	O157 : H-	*stx1*, *stx2, espP*	Human	[60]
E2348/69	O127 : H6	*eae*, *espB*, *bfpA*	Human	[61]
H10407	O78 : (K80) : H11	*cfa*, *est-Ia*, *est-Ib*, *elt-Ib*	Human	[62]
HUS-2/85	O111 : H-	*stx1*	Human	[63]
BM2-10	O88 : H25	CNF2	Calf	[64]
E57	O138 : K81	*stx2e, est-Ia*	Swine	[65]
F107/86	O139 : K12 : H1	*fedA, stx2e*	Swine	[66]
17-2	O3 : H2	*aaf, astA*	Human	[67]
Abbotstown	O149 : K91	*fae, elt-Ib, elt-Ia, est-II*	Swine	[68]
B41	O101 : H-	F5, F41, *fan, elt-Ia*	Cattle	[69]
EDL933	O157 : H7	*stx1*, *stx2*, *eae*, hly_{EHEC}	Human	[70]
E38	O78 : H-	n.n.	Human	[71]
Shigella flexneri 2A				
2457T		*ipaC*		Kindly provided by A. Fasano, University of Maryland, Baltimore

to the already known zoonotic potential of septicemic O78 strains in humans [9, 15], the strains were also screened for virulence genes associated with common factors of pathotypes causing diarrhea or septicemia in humans.

2 Materials and methods

2.1 Bacterial strains and culture conditions

A total of 21 *E. coli* O78 strains originating from fecal samples of 21 diarrheal calves and cattle over a period of ten years were studied. Fecal samples were sent to the diagnostic laboratories of the veterinary faculties from Giessen and Berlin, Germany, for diagnostic purposes. The diseased animals originated from a total of ten farms in Hessia, North Rhine-Westphalia, Lower Saxony, Bavaria, Mecklenburg-Western Pommerania, and Brandenburg, respectively. *E. coli* strains used as controls in PCR- and DNA-DNA-hybridization analyses are listed in Table 1. For cell adherence and invasion assays, control bacteria were propagated on brain heart infusion agar. *Salmonella* (*S.*) *enterica* ssp. *enterica* serovar Typhimurium strain SL 1344 (kindly provided by B. Brett Finlay, University of British Columbia, Vancouver) was used as positive control (invasive), and Δ *inv*A *S. enterica* ssp. *enterica* serovar Typhimurium strain SB 147 (kindly provided by Jorge E. Gálan, Stony Broke, New York) as negative (noninvasive) control in invasion assays. Stock cultures from all reference and control strains were stored at −80°C in brain heart infusion (BHI) broth with 15% glycerol and streaked onto BHI agar plates prior to inoculation of the working cultures.

2.2 Cell lines and cell culture

Monolayers of MDBK (derived from a bovine kidney carcinoma)-cells (ATCC-No. CCL22) were grown in 24-well cell culture plates at 37°C in 5% CO_2, using RPMI 1640 (40%; Life Technologies, Karlsruhe, Germany) containing fetal bovine serum (10% FBS; Sigma, Deisenhofen, Germany), L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies) cell-growth medium.

2.3 Adherence and invasion assays

The bacterial invasion of epithelial cells was tested in the gentamicin protection assay [16]. The minimal bactericidal concentration (MBC) of gentamicin was determined in tissue culture medium, and the antibiotic was used at 10- to 100-fold the MBC (MBC ≤ 1µg/mL). Since similar results were obtained independent of the gentamicin concentration even for prolonged periods of up to 24 h, a final concentration of 100 µg/mL was used in subsequent experiments. Monolayers were seeded in two 96-well tissue culture dishes (Greiner, Frickenhausen, Germany) per experiment with 3×10^4 cells/well for MDBK cells and incubated for 48 h. The cell monolayers were washed twice with phosphate-buffered saline (PBS, pH 7.2). Exponential-phase bacteria were grown in Luria Bertani (LB) broth at 37°C, washed, and resuspended in PBS at a densitiy of 3×10^7/mL. Each monolayer was infected at a multiplicity of infection (MOI) of 100 bacteria per epithelial cell. After a 3 h incubation period at 37°C the bacterial supernatant of plate I was collected for bacterial cell counting and infected monolayers of both plates were washed three times with PBS. For measurement of bacterial invasion fresh culture medium containing 100 µg of gentamicin per mL was added to cell culture plate I to kill extracellular bacteria. After incubation for additional 1.5 h, monolayers of plate I were washed three times with PBS, and 200 µL of Triton X-100 (Sigma, Deisenhofen, Germany) in 1% PBS was placed in each well of both cell culture plates for 5 min to

Table 2. Sequence, specificity, and PCR conditions applied for the detection of virulence-associated genes in *E. coli* strains

Primer	Primer sequence (5′ – 3′)	Detected gene	Location within gene	Acc. No.	PCR conditions (°C/s)[a]			Ref.
					Denaturation	Annealing	Elongation	
EAST-1 s	TGCCATCAACACAGTATATCC	*astA*	135 – 155	AF143819	94/40	50/80	72/60	[20]
EAST-1 as	TAGGATCCTCAGGTCGCGAGTGACGGC		219 – 245					
FyuA s	GCGACGGGAAGCGATGACTTA	*fyuA*	786 – 806	Z38064	94/60	65/60	72/60	[52]
FyuA as	CGCAGTAGGCACGATGTTGTA		1539 – 1559					
HMWP-2 s	AAGGATTCGCTGTTACCGGAC	*irp2*	22 – 42	L18881	94/60	55/60	72/60	[52]
HMWP-2 as	TCGTCGGGCAGCGTTTCTTCT		434 – 416					
AERA s	ACAAAAAGTTCTATCGCTTCC	*iucD*	239 – 259	M18968	94/60	55/60	72/60	[23]
AERA as	CCTGATCCAGATGATGCTC		913 – 931					
EspP s	TTGCGAAAAATGGCGGAACTC	*espP*	1418 – 1438	Y13614	94/60	55/90	72/90	[18]
EspP as	CGGAGTCGTCAGTCAGTAGA		2374 – 2355					
ECW1 s	TGCGGCACAACAGGCGGCGA	*eae*	563 – 582	Z11541	94/60	68/90	72/90	[53]
ECW2 as	CGGTCGCCGCACCAGGATTC		1191 – 1172					
EHLY1 s	GAGCGAGCTAAGCAGCTTG	*hlyEHEC*	949 – 967	X94129	94/60	63/90	72/90	[54]
EHLY5 as	CCTGCTCCAGAATAAACCACA		1838 – 1818					
HLYA s	GTCCATTGCCGATAAGTTT	*hlyA*	972 – 991	M14107	94/40	50/80	72/60	This study
HLYA as	AAGTAATTTTTGCCGTGTTTT		1322 – 1302					
F5 s	TATTATCTTAGGTGGTATGG	*f5*	90 – 109	M35282	94/30	50/45	72/90	[55]
F5 as	GGTATCCTTTAGCAGCAGTATTTC		403 – 380					
F41 s	GCATCAGCGGCAGTATCT	*f41*	288 – 305	X14354	94/30	50/45	72/90	[55]
F41 as	GTCCCTAGCTCAGTATTATCACCT		667 – 644					
Tia-I s	GCGGTTTCAGCGCTTGCA	*tia*	25 – 42	U20318	94/60	54/90	72/90	[56]
Tia-II as	GTTACCCCAAGCATAATGTC		743 – 724					
IpaC-I s	ATCATTGCTCGCCTTACTGAC	*ipaC*	3054 – 3074	M34849	94/60	54/90	72/90	[56]
IpaC-II as	GCAATCTGACTGGCTGCCG		3913 – 3895					
MK1 s	TTTACGATAGACTTCTCGAC	*stx*	115 – 134	AY143336	94/40	50/80	72/60	[57]
MK2 as	CACATATAAATTATTTCGCTC		300 – 280	AB048222				
ST-IB s	CCTGACTCTTCAAAAGAGAAAATTAC	*est-Ia/est-Ib*	360 – 383	M25607	94/40	50/80	72/60	[58]
ST-IC as	GATTACAACAAAGTTCACAGCAGT		481 – 458					
LT-I1 s	TCTCTATGTGCATACGGAGC	*elt-Ia/elt-Ib*	250 – 269	J01646	94/40	55/80	72/60	[59]
LT-I2 as	CCATACTGATTGCCGCAAT		571 – 553					
CNF-1/2 s	TCGTTATAAAATCAAACAGTC	*cnf-1*	1817 – 1846	X70670				
			2262 – 2241		94/50	50/80	72/60	This study
CNF-1/2 as	CTTTACAATATTGACATGCTG	*cnf-2*	1073 – 1093	U01097				
			1518 – 1498					
CDT-IIIB s	TTTTTATCGTTTTACGCTCAG	*cdt-IIIB*	1885 – 1905	U89305	94/50	50/80	72/60	This study
CDT-IIIB as	CGCGATTAAAATCACCAA		2440 – 2423					
Cva A/B	TGGTAGAATGTGCCAGAGCAAG	*cva A/B*	10745 – 10764	AJ223631	94/30	57/30	72/90	[17]
Cvi CvaC	GAGCTGTTTGTAGCGAAGCC	*cvi/cvaC*	11925 – 11904					

a) Initial denaturation step for all protocols: 94°C, 3 min; final elongation: 72°C, 10 min

lyse eukaryotic cells. This concentration of Triton X-100 did not affect bacterial viability even after 30 min (data not shown). Samples were removed, and serial 10-fold dilutions were plated. Invasion levels were expressed either as the number of Colony-forming units (CFU) recovered per well after treatment with gentamicin or as the percentage of the original inoculum resisting treatment with gentamicin. To determine the total number of cell-associated bacteria corresponding to adherent and intracellular bacteria, the eukaryotic cells of plate II were lysed after the 3 h infection period and bacteria were quantified as described above. All assays were conducted in duplicate and were independently repeated at least four times. Results are expressed as the averages of all replicate experiments ± standard deviations.

2.4 PCR and hybridization experiments

The *E. coli* strains were analyzed by PCR and DNA-DNA-hybridization (dot blot) analyses for the presence of 18 virulence associated genes listed in Table 2. Bacterial DNA was released from whole organisms by boiling, and PCR as well as DNA-DNA-hybridization analyses were performed as described [17].

2.5 Contour clamped homogeneous electric field-plused field gel electrophoresis

Genomic DNA for contour-clamped homogeneous electric field-pulsed field gel electrophoresis (CHEF-PFGE) was extracted as recently described [18]. Slices of DNS containing agarose plugs were incubated for 4 h with 20 U of *Xba*I (Oncor Appligene), and *Bln*I (Roche Diagnostics, Mannheim, Germany), respectively. The respective DNA fragments were separated by agarose gel electrophoresis (1% agarose gel) (pulsed-field certified agarose; Bio-Rad, München, Germany) in a CHEF-DR-III-system (Bio-Rad) at 6 V/cm with 0.5% Tris-borate-EDTA as a running buffer. The pulsed-field times for both enzymes were increased from 5 to 50 s during the running time of 22 h at 14°C (angle: 120°C, ramping: linear). Polymerized phage DNA (Promega, Mannheim, Germany) served as a size standard. CHEF-PFGE patterns between these sizes were analyzed

with GelCompar® (Applied Maths BVBA, Kortrijk, Belgium; Herolab) as described previously [18].

2.6 Statistical analysis

For interpretation of gentamicin protection assay, data were transformed logarithmically. We calculated standard deviation values for the transformed data and listed results as dislogarithmic data. In the case of $p < 0.05$ results were compared with the Dunnett-T-Test against reference *E. coli* O78 strain, as well as the invasion-deficient *S.* Typhimurium mutant.

3 Results

3.1 Detection of virulence-associated genes

21 *E. coli* field strains of serogroup O78 were investigated for the prevalence and distribution of 18 virulence genes associated with adhesion, toxicity, and invasion not only in bovine but also in human *E. coli* pathotypes by both PCR and DNA-DNA-hybridization analysis (Table 2). None of the 21 bovine *E. coli* serogroup O78 strains harbored the enterotoxin genes *estIa/Ib*, *eltIa/Ib*, or the fimbria-associated genes *f5* and *f41* that are commonly distributed in ETEC strains isolated from calves (Table 3). We also did not detect *eae* (attaching and effacing), coding for the adherence determinant initimin typically found in enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli*. Among the O78 strains tested there was no isolate positive for genes *stx* and hly_{EHEC}, whereas five strains (23.8%) harbored the protease gene *espP* (syn. *pssA*). Invasion gene *tia*, that has been described in ETEC strains, was present in five (23.8%) O78 strains but could only be detected by DNA-DNA-hybridization. Virulence genes related to enteroaggregative *E. coli* (EAEC) were often found. The heat stable enterotoxin-1 (EAST-1)-encoding *ast*A was detected in more than half of the isolates (61.9%), as well as *fyuA* and *irp2*, encoding important components of an iron acquisition system (each 57.1%). However, we could not detect any strain positive for the hemolysin gene *hly*A which is also commonly found in strains belonging to EAEC.

The aerobactin-encoding gene *iucD* was present in 17 (81.0%) O78 strains and thus revealed the highest prevalence among the virulence-associated genes investigated in this study. Eight (38.1%) strains harbored genes constituting the operon of the ColicinV-plasmid that is highly distributed in *E. coli* strains isolated from extraintestinal diseases.

The genes *cdt-IIIB* und *cnf-1/2*, both encoding cytotoxins of necrotoxic *E. coli* (NTEC), were present in 3 (14.3%) strains and always in combination. The invasion-associated gene *ipaC* that is common in enteroinvasive *E. coli* (EIEC), was not found among the 21 O78 strains. The gene combination with the highest prevalence in O78 strains was *fyuA*, *irp2*, *iucD*, and *astA* in about half of the strains tested (47.6%). Five of the strains (23.8%) harbored a combination of the genes *astA*, *fyuA*, *irp2*, *iucD*, *tia*, and *colV*. Two (9.5%) of the strains tested did not possess any of the 18 genes we screened for. As outlined in Table 3, with the

Table 3. Virulence patterns of wild-type *E. coli* O78-strains ($n = 21$) as determined by PCR and DNA-DNA-hybridization

Positive strains (%)	*stx*	*astA*	*cdt-IIIB*	*cnf1/cnf2*	*estIa/estIb*	*eltIa/estIb*	*fyuA*	*irp2*	*iucD*	*hlyA*	*hly EHEC*	*eae*	*f5*	*f41*	*espP*	*tia*	*ipaC*	*colV*
	0	61.9	14.3	14.3	0	0	57.1	57.1	81.0	0	0	0	0	0	23.8	23.8	0	38.1
Strain																		
IMT818	–	–	–	–	–	–	+	+	+	–	–	–	–	–	+[a)]	–	–	–
IMT2413	–	–	–	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
IMT832	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	+[a)]	–	+
IMT833	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	+[a)]	–	+
IMT837	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	+[a)]	–	+
IMT840	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	+[a)]	–	+
IMT844	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	+[a)]	–	+
IMT839	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	+
IMT1929	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	+
IMT1923	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
IMT1928	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
IMT1413	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
IMT1520	–	+	–	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
IMT1521	–	+	–	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
IMT5468	–	+	–	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
IMT2513	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
IMT5469	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+
IMT5174	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IMT803	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IMT823	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IMT2409	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+ or – indicates the presence or absence of this genotype

a) PCR-negative, DNA-DNA-hybridization-positive

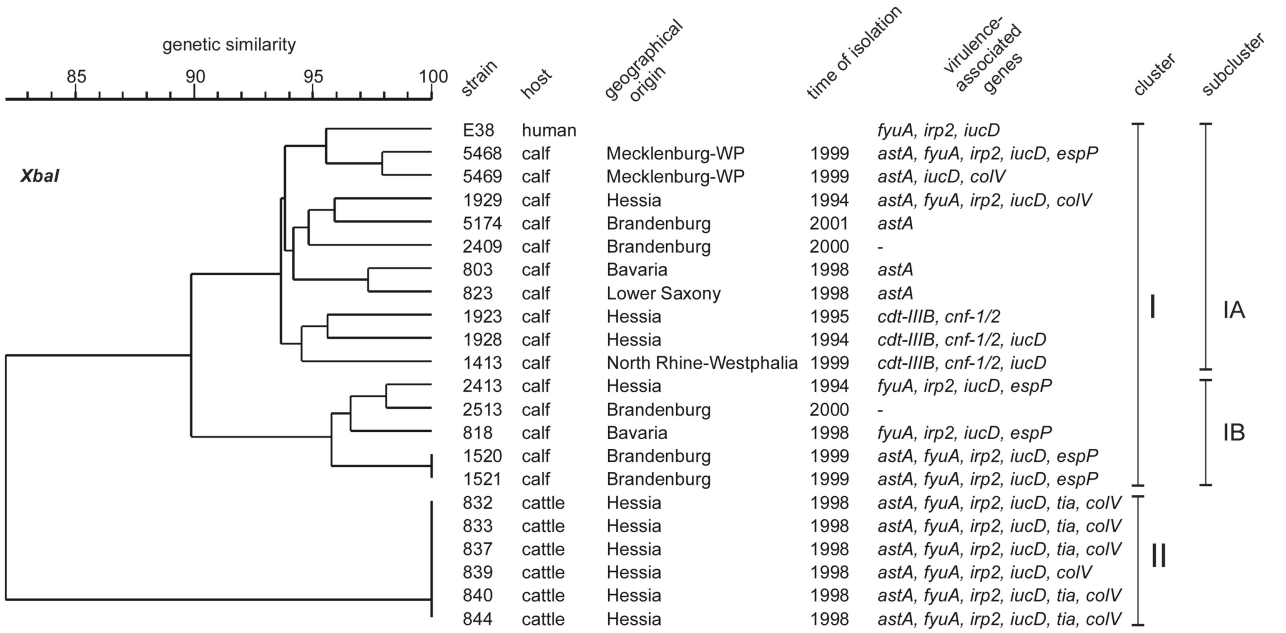

Figure 1. Grouping of bovine *E. coli* serogroup O78-isolates according to genetic similarity after *Xba*I-restriction and association to virulence genes.

exception of all 5 *tia* positive strains and one *espP*-positive strain, each time PCR results were confirmed by DNA-DNA-hybridization.

3.2 Clonal analysis

All strains were analyzed by PFGE utilizing *Xba*I and *Bln*I, and compared with human *E. coli* O78 reference strain E38. After restriction with *Xba*I the 21 *E. coli* O78 strains grouped into clonal cluster I and II with a similarity of 82.1%. Cluster II (6 strains) revealed an internal similarity of nearly 100%, whereas cluster I split into subclusters IA and IB, showing a within-similarity of 89.9%. Both subclusters revealed an internal similarity of 93.7% (IA) and 95.8% (IB), respectively. Subcluster IB contained two strains with identical restriction patterns (Fig. 1).

Similarly, *Bln*I-restriction resulted in two major clonal groups differing 26.0% to each other and with internal similarities of 89.0% (cluster I) and 99.3% (cluster II), respectively. Cluster I could further be divided into two subclusters with an internal similarity of 93.4% (IA) and 94.6% (IB). Thus, restriction analyses with two different endonucleases *Xba*I and *Bln*I gave nearly identical results of clonal relationship, revealing cluster II to be one clone (Fig. 2).

An association between clonal relationship and virulence gene patterns could be detected in cluster II and subcluster IB (Figs. 1 and 2). Virulence-associated genes *astA*, *fyuA*, *irp2, iucD*, and *colV* were present in 100%, *tia* in 83.3% of strains belonging to cluster II. 80% of strains grouped into subcluster IB possessed *fyuA*, *irp2*, *espP*, and *iucD*, and half of them additionally harbored *astA*. All three isolates that harbored cytotoxin genes *cdt-IIIB* and *cnf-1/2* belonged to subcluster IA. In this subcluster, virulence-associated genes *astA* (60.0%), *fyuA* and *irp* (each 20.0%), *espP* (10.0%), *iucD* (50.0%), and *colV* (20.0%) were present with a lower frequency compared to cluster II.

3.3 Adherence and invasion assay for bovine *E. coli* of serogroup O78

Adhesive and invasive virulence properties are important features during intestinal infections. We therefore investigated the ability of bovine O78 strains for adherence and invasion in a quantitative gentamicin protection invasion assay. MDBK-cell monolayers were incubated with *Salmonella* control strains and three different gentamicin resistant *E. coli* O78 isolates varying in their virulence gene pattern. Strains used in these assays were IMT803 (*astA*), IMT1928 (*cdt-IIIB*, *cnf-1/2*, *iucD*), and IMT2413 (*fyuA*, *irp2*, *iucD*, *espP*). The human *E. coli* O78 reference strain E38 (*fyuA*, *irp2*, *iucD*) was also included for comparative analyses, and in addition, no data concerning these properties were available.

3.3.1 Adhesiveness

Like *Salmonella* control strains *E. coli* serogroup O78 isolates adhered to MDBK-cells with various efficiencies (Fig. 3). The number of cell-associated bacteria ranged

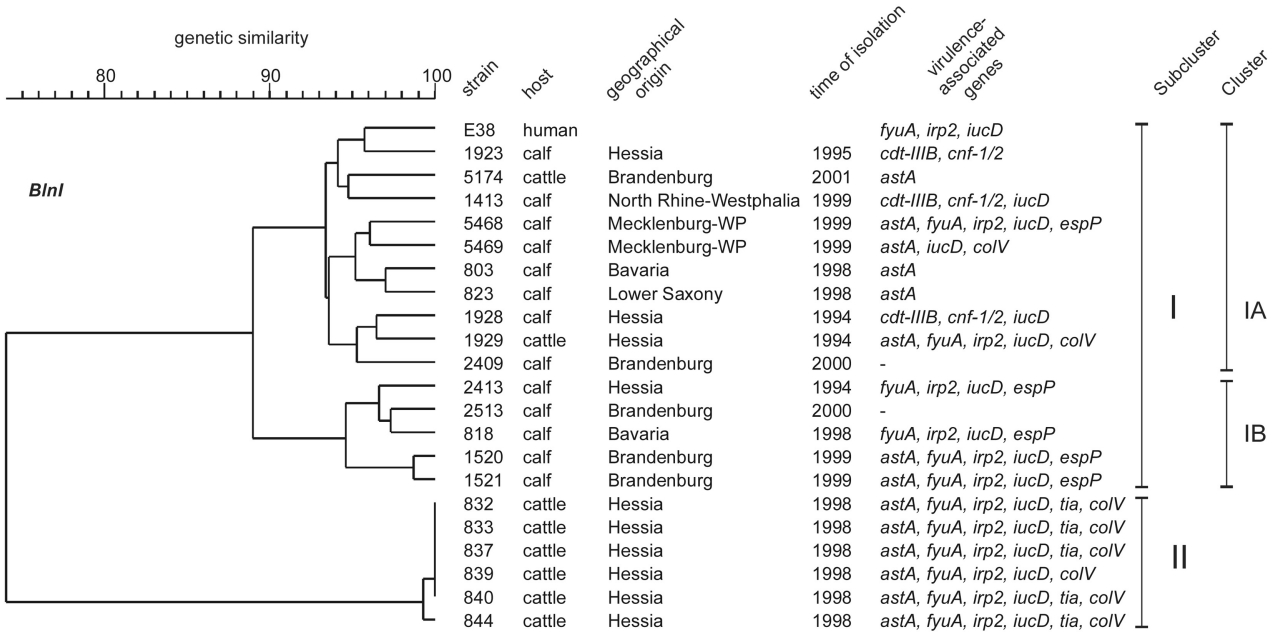

Figure 2. Grouping of bovine *E. coli* serogroup O78-isolates according to genetic similarity after *Bln*I-restriction and association to virulence genes.

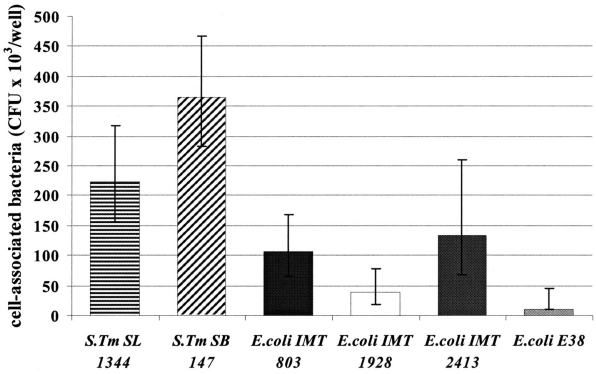

Figure 3. Adhesiveness of *Salmonella*- and *E. coli*-strains on MDBK-cells (gentamicin protection assay, MOI 100, 3 h incubation).

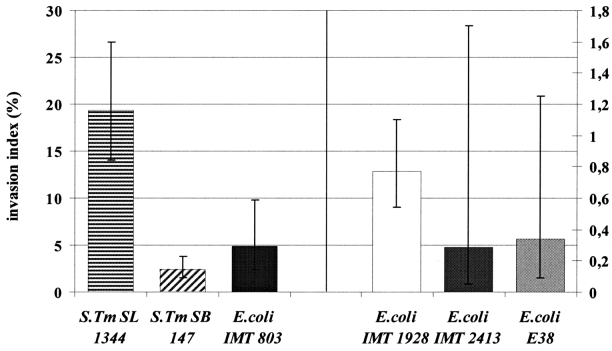

Figure 4. Invasiveness of *Salmonella*- and *E. coli*-strains in MDBK-cells (gentamicin protection assay, MOI 100, 3 h incubation without gentamicin followed by 1.5 h incubation with gentamicin).

between 10.77×10^3 CFU/well (*E. coli* E38) and 364×10^3 CFU/well (*S.* Typhimurium SB 147) resembling 0.36 to 12.1 cell-associated bacteria per initially seeded epithelial cell. Invasive control strain *S.* Typhimurium SL 1344 showed an adherence of 222.35×10^3 CFU/well. Among O78 isolates tested *E. coli* strain IMT2413 adhered most intensely to MDBK-cells (132.58×10^3 CFU/well). IMT803 and IMT2413 were significantly more adhesive than human O78 reference strain E38 ($p < 0.05$). In contrast the differences in adherence between *E. coli* strains E38 and IMT1928 were significantly lower compared to the invasion-deficient *S.* Typhimurium mutant SB 147 ($p < 0.05$).

3.3.2 Invasiveness

MDBK-cells were invaded by all investigated strains with positive control strain *S.* Typhimurium SL 1344 revealing the highest invasion index (19.32%), and *E. coli* strain IMT2413 showing the lowest invasion index of 0.29% (Table 4, Fig. 4). The Δ*invA*-mutant *S.* Typhimurium SB 147 showed an invasion index of 2.38%. *E. coli* O78 wild-type strain IMT803 had a rather low invasion index with 4.88% that, however, was still higher than that of the invasion-defective mutant *S.* Typhimurium SB 147, whereas *E. coli* strains E38, IMT1928, and IMT2413 showed lower invasion indices. Significant differences in invasion proper-

Table 4. Invasiveness of bacterial strains in MDBK-cells

Bacterial strain	Number of intracellular bacteria (CFU/well × 10^3)		Invasion indices (%)	
	x	Standard deviations	*x*	Standard deviations
S. Tm SL1344	42.96	25.370–72.75	19.32	14.03–26.60
S. Tm SB147	8.68	6.680–11.28	2.38	1.50–3.79
E. coli E38	0.037	0.032–0.041	0.34	0.09–1.25
E. coli IMT803	5.15	3.260–8.14	4.88	2.44–9.76
E. coli IMT1928	0.3	0.110–0.82	0.77	0.54–1.1
E. coli IMT2413	0.38	0.036–3.97	0.29	0.05–1.70

x: values shown are the averages of at least four (eight for *S.* Typhimurium SL 1344) independent experiments conducted in duplicate ± standard deviations.

ties were determined between IMT2413 and Δ*inv*A-mutant *S.* Typhimurium SB 147, as well as for IMT803 and O78 reference strain E38 ($p < 0.05$). Numbers of intracellular bacteria are listed in Table 4. Values ranged between 0.037×10^3 CFU/well (*E. coli* E38) and 42.96×10^3 CFU/well (*S.* Typhimurium SL 1344), thus between 0.001 and 1.43 invaded bacteria per initially seeded MDBK-cell.

4 Discussion

An increasing isolation rate of O78 strains from diarrheal cases in bovines prompted us to investigate the pathogenic, clonal and presumably zoonotic potential of these strains, which currently can not be defined to any of the established *E. coli* pathotypes [4]. By means of PCR- and DNA-DNA-hybridization analyses of 21 O78 strains, isolated from diarrheal cases in bovines, we found the toxin gene *astA*, which is a major characteristic of the pathotype enteroaggregative *E. coli* (EAEC), with high prevalence in our strains. The wide distribution of enteroaggregative heat-stable enterotoxin-1 (EAST-1) in different *E. coli* pathotypes isolated from intestinal and various extraintestinal disease as well as from apathogenic strains is due to its localization on a transposon [19–23]. The function of EAST-1 in the pathogenesis of intestinal diseases is not well explored, but it is supposed that toxin expression alone is not sufficient to cause disease [24].

There was a striking distribution of genes involved in iron acquisition among O78 strains. Two different systems could be detected with *fyuA* and *irp2* coding for the *Yersinia*-specific siderophore yersiniabactin located on a high pathogenicity island, and *iucD* encoding the hydroxamate type siderophore aerobactin. Both siderophores are widely distributed in human septicemic and enteroaggregative *E. coli* as well as in avian septicemic *E. coli* [23, 25–28]. More than half of the O78 strains (57.1%) possessed genes coding for both yersiniabactin and aerobactin. It is well known, that iron is not only a nutritional source for bacteria but also influences cellular immunity of the host, and thus iron-scavenging contributes to virulence, especially in extraintestinal diseases. It is supposed that aerobactin either influences the extent of bacterial translocation in the intestine by providing high colonization efficiencies or bacterial growth in tissue after translocation or both [29].

Eight of the investigated strains also harbored the operon sequence of colicin V (ColV)-plasmids. These plasmids encode virulence features like aerobactin, increased serum survival, and bacterial resistance against phagocytosis [30]. It has been suggested that in ColV-producing intestinal *E. coli*, these genes are located on the chromosome, whereas in isolates from blood they are located on plasmids. ColV-plasmids are common virulence determinants of uropathogenic and avian pathogenic *E. coli*, both important extraintestinal pathogens [13, 31, 32]. Furthermore, there is a difference in virulence patterns of O78 isolates from sporadic cases of diarrhea and septicemia and those from healthy individuals. While epidemic strains and those pathogenic for calves produced colicin V, the latter strains produced other colicin variants or no colicins at all [33].

In three strains, we detected genes encoding cytotoxic necrotizing factor 1 and 2 (*cnf1/2*) and cytolethal distending toxin III (*cdt-IIIB*) always in combination. *Cnf2* and *cdt-IIIB* are located on a Vir-plasmid coding for a toxin which causes cytopathic effects on HeLa-cells and in addition a pilus-like surface antigen [34–36]. The expression of CNF is a typical feature of necrotoxigenic *E. coli* (NTEC). CNF2-producing *E. coli* (NTEC-2) have been isolated from intestinal and extraintestinal infections of ruminants, especially from bovine extraintestinal strains of serogroup O78. They are also widely distributed in healthy cattle and calves [37–40]. Experimental infections of calves with NTEC-2 have shown that these bacteria are able to colonize the intestine and enter the bloodstream, but their potential to cause clinical symptoms strongly depends on the hosts immune status, and NTEC-2 thus are supposed to be opportunistic pathogens [5]. We were not able to identify genes coding for both ColV- and Vir-plasmid-determinants which

is in accordance with other studies on bovine O78 strains [9, 41]. Possession of *cnf1/2* and *cdt-IIIB* gains further importance by recent findings of these toxins in non-O157 enterohemorrhagic *E. coli* [42].

Some of the O78 strains investigated in our study possessed the episomal gene *espP* (for extracellular serine protease P) coding for a secreted protease of the family of autotranporter proteins. EspP, which is found in EHEC and EPEC strains, is suggested to act against cytosceletal proteins, causing direct damage of epithelial cells in the intestine, indirectly facilitating bacterial entrance to these cells, which are covered by mucus [43 – 45].

The gene *tia* (for toxigenic invasion locus), that was present in 23.8% of our strains, has been predominantly detected in ETEC strains. Tia functions both as adhesin and invasin and resembles a new type of afimbrial adhesin [46]. As in other studies analysing the presence of *tia* in invasive and intestinal *E. coli* we only detected this gene by DNA-DNA-hybridization, but not by PCR. Like others, we suppose, that the *tia* locus has striking similarities to other loci not yet been determined and also present in our strains [47].

Our results of PCR- and DNA-DNA-hybrization analyses illustrate, that more than half of the strains cannot be differentiated from septicemic O78 strains by their genotype, and in future studies it has to be shown whether *E. coli* O78-strains isolated from intestinal diseases constitute an independent intestinal population, able to cause diarrhea.

Previous studies gave contradictory results about the clonal relatedness of septicemic *E. coli* serogroup O78 strains isolated from humans and animals worldwide [9, 10, 12, 48]. As shown recently by multilocus sequence typing (MLST), O78 clones are host independent, and closely related clones reside in different hosts. These authors also found a positive correlation between virulence and clonal origin [7]. Although analyzing only 19 O78 strains, 14 different sequence types were identified. These findings are of major importance, as they clearly show, that O78 strains represent various independent clones. Thus, a comparative analysis of the genomic relationship between O78 strains isolated from intestinal diseases and O78 strains from other diseases in man and animals is urgently needed to give a sound and valid assessment of the zoonotic potential of these strains. Macrorestriction analyses performed in our study revealed that the clonal relatedness of diarrhaegenic O78 strains is just as close as that of septicemic strains belonging to this serogroup. Thus, bovine *E. coli* O78 strains isolated from diarrheal cases presumably harbor a zoonotic potential, especially as they are more widely spread through faeces, contaminating the environment.

Adherence of microorganisms to host cells is the first step during infection of the host, being essential not only in the pathogenesis of intestinal diseases [49, 50]. Moreover, the ability to invade epithelial cells often is an important virulence mechanism in the pathogenesis of *E. coli* infections [50]. *E. coli* serogroup O78 strains typically cause septicemia and thus are supposed to have adhesive as well as invasive potential. Concerning theses virulence mechanisms there are no data for O78 strains isolated from diarrheal cases in cattle so far.

All O78 strains investigated in this study including the human O78 reference strain E38 were able to adhere to and invade MDBK-cells with different efficiencies. As adhesion and invasion are likely processes underlying interactions between bacteria and host-specific receptors the low adherence and negligible invasion level of human reference strain E38 has to be assessed critically as we used a bovine eptithelial cell line. It has been shown that pathogenic bacteria secreting members of the familiy of autotransporters had striking abilities to colonize intestinal or respiratory mucosal surfaces [43]. This is confirmed by the strong adherence property of strain IMT2413, which harbors the serine protease-encoding gene *espP*. The cytotoxic effect of EspP could contribute to the low invasion index of this strain, as other studies have shown that this protease causes structural damages in Vero cells after short-time-incubation periods [43]. CNF-1-producing *E. coli* are supposed to possess a loose adhesion-phenotype, which does not allow the bacteria to resist repeated washings or gentamicin treatment [51]. This could explain moderate adherence levels of CNF-1/2- and CDT-IIIB-producing strain IMT1928 to MDBK-cells followed by low invasion efficiencies. With an invasion index of 25% of that of control strain *S.* Typhimurium SL1344, *E. coli* IMT803 revealed the strongest invasiveness among bovine O78 strains tested. As this strain only harbors *astA* we suppose that there have to be additional virulence associated factors coding for adherence and invasion properties of this isolate.

Adhesion and invasion assays confirmed similar virulence properties of O78-strains isolated from bovine diarrhea and septicemic strains of this serogroup. Further studies have to be performed as our assays were limited to a single cell line that differs from native cells in the intestine concerning surface structures like receptors and the density of these surface structures as well as mucus production.

The data presented here confirm that *E. coli* O78 strains harbor virulence-associated genes, the gene products of which are able to cause intestinal disease in cattle. They possess numerous virulence genes associated with intestinal *E. coli* pathotypes but cannot be grouped definitively to one of those established pathotypes. *E. coli* serogroup O78 strains seem to have the ability to gain and express several virulence properties, and act bifunctional as causative agents of septicemic as well as intestinal infections. Our

cumulative data indicate that O78 strains isolated from diarrhaegenic cattle and calves may have zoonotic potential, urging further investigations on this topic.

5 References

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