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Assessment of Adhesins as an Indicator of Pathovar-Associated Virulence Factors in Bovine *Escherichia coli*

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The CS31A, F17, and F5 adhesins are usually targeted by serology-based methods to detect pathogenic *Escherichia coli* associated with calf enteritis. However, the virulence traits of the selected isolates are still poorly described. Here, from a set of 349 diarrheagenic *E. coli* isolates from cattle, we demonstrated a 70.8% concordance rate (Cohen's kappa, 0.599) between serology- and PCR-based approaches for the detection of adhesins under field conditions. A 79% to 82.4% correspondence between the two methods was found for fimbrial adhesins, whereas major discrepancies (33%) were observed for CS31A-type antigens. Various F17A variants were found, such as F17Ac (20K) (50%), F17Aa (FY) (18.9%), F17Ab (8.1%), and F17Ad (111K) (5.4%), including a high proportion (17.6%) of new F17A internal combinations (F17Aab, F17Aac, and F17Abc) or untypeable variants. In addition, the highest proportion of pathovar-associated virulence factor (VF) genes was observed among *E. coli* isolates that produced F5/F41 adhesins. A specific link between the heat-stable toxins related to the enterotoxigenic *E. coli* (ETEC) pathovar and adhesins was identified. STa was significantly linked to F5/F41 and EAST1 to CS31A adhesins ($P < 0.001$), respectively, whereas NTEC was associated with F17 adhesin ($P = 0.001$). Clustering between phylogroups according to the adhesin types was also observed. Also, few Shiga toxin-producing *E. coli* (STEC) or enteropathogenic *E. coli* (EPEC) pathovars were identified. Finally, no statistically significant difference was observed in the occurrence of extended-spectrum beta lactamase (ESBL) production according to the adhesins expressed by the isolates ($P = 0.09$). Altogether, this study gives new insights into the relationship between adhesins, VF, and antimicrobial resistance in calf enteritis and supports the need for further standardization of methodologies for such approaches.

Escherichia coli is a common enteric pathogen in humans and animals, and serological typing of O antigens, such as O157, O26, O103, O111, and O145, is useful for the identification of dominant *E. coli* strains associated with human disease (1). On the other hand, many other serotypes or untypeable *E. coli* isolates are involved in animal diseases, and they cannot be analyzed routinely by veterinary laboratories. Other methods have been developed for such veterinary purposes, among which are coagglutinating reagents against specific adhesin antigens designed for the detection of *E. coli* isolates associated with calf enteritis (2). The F17, F5 (K99), and F41 fimbriae and the afimbrial CS31A adhesin are usually targeted. Indeed, F17, F5, and F41 were mainly associated with enterotoxigenic *E. coli* (ETEC) in calves (3). The F17Ac (20K) subtype is the prominent adhesin among bovine septicemic *E. coli* isolates and was also found in human necrotoxic *E. coli* (NTEC) isolates, whereas the F17Ab subtype was often associated with ruminant NTEC (4–6). Some data suggest a close association of CS31A-producing *E. coli* with cases of septicemia (7), and CS31A was also associated with cytotoxic necrotizing factor 1 (CNF1) among bovine-pathogenic *E. coli* strains (8, 9).

As the serotype and/or adhesin production does not necessarily reflect the virulence traits, these adhesin- or serogroup-based diagnostic tools allow the identification of only a limited number of pathogenic *E. coli* strains. Thus, to prevent zoonotic and foodborne diseases caused by *E. coli*, the relevance of the current diagnostic methods for rapid identification of pathogenic *E. coli* at the farm level needs to be investigated. In addition, as adhesins can be used as immunogens, better knowledge of the virulence factors (VFs) associated with adhesins is of interest for vaccine development. Finally, antimicrobial resistance has now become an important bacterial trait in animals, and few data are available on the codistribution of resistant determinants, such as those to broad-spectrum cephalosporins, and adhesins and/or VFs in pathogenic *E. coli* strains from cattle (10–12).

The first objective of this work was to assess the correlation between serology- and PCR-based methods for the detection of adhesins. Then, we evaluated whether the presence of adhesins in bovine-pathogenic *E. coli* isolates is a relevant indicator of the presence of pathovar-associated VFs, as routinely inferred by veterinary laboratories. We also investigated any correlation between the presence of adhesins and/or VFs and the distribution of genes involved in the resistance to broad-spectrum cephalosporins.

MATERIALS AND METHODS

***E. coli* isolates.** A total of 349 *E. coli* isolates from diseased or dead calves (diarrhea, $n = 259$; septicemia, $n = 13$; dead, $n = 31$; and not defined, $n = 46$) in 2011 and 2012 were collected from 29 different geographic areas

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(districts) throughout France and selected on the basis of CS31A, F17, F5, or F41 adhesin detection, as determined with serology-based methods by qualified veterinary laboratories. Each sample was nonreplicate, i.e., only one sample per farm was included in this study.

Confirmation of adhesins, *E. coli* phylogrouping, VF genes, and antibiotic susceptibility testing. Isolates were identified using colony morphology and API20E tests (bioMérieux, Marcy l'Etoile, France). Extraction and purification of DNA were performed using the DNeasy blood and tissue kit (Qiagen, France). The presence of the adhesin genes and their variants was confirmed using PCR, as previously described (8, 13, 14). For isolates that tested negative for all F17A variants or positive for more than one F17A variant, F17A-derived PCR products (13) were sequenced by Beckman Coulter, London, United Kingdom. Only isolates providing PCR results confirming the diagnosis obtained by veterinary laboratories were further considered to evaluate the presence of a first set of VF-encoding genes (listed in Table S1 in the supplemental material). The reference strains previously described (15) were used as positive controls for the detection of *aggR* (17-2), *bfpA* (E2349/19), and *stb* (987) genes. *E. coli* isolates were assigned to phylogroups using the revisited Clermont *E. coli* phylotyping method (16), and their susceptibilities to 16 β-lactams was determined by disc diffusion according to the guidelines of the Antibiogram Committee of the French Society of Microbiology (CA-SFM [www.sfm-microbiologie.org]). The $bla_{\text{CTX-M}}$ genes were detected in all extended-spectrum beta-lactamase (ESBL) producers using a CTX-M group-specific multiplex PCR (17). To precisely define the virulotype, positive isolates presenting at least one of the above-mentioned virulence markers were further analyzed by PCR using a second set of primers (see Table S1 in the supplemental material). For Shiga toxin-producing *E. coli* (STEC), the subtypes of stx_1 and stx_2 genes, the *eae* variants, and the five main serogroups and their associated flagellar (H) antigens were determined as previously described (18, 19).

Statistical analysis. To compare serology- and PCR-based methods, Cohen's kappa coefficient was calculated with a 95% confidence interval. Comparison of proportions was performed using the chi-square test or the Fisher exact test, if needed (for very low proportions). The threshold value (*P* value) was 0.05.

Nucleotide sequence accession numbers. The 6 F17A untypeable (F17AND) variant-derived PCR sequences using P1 and P2 primers (13) were deposited in the NCBI database under GenBank accession numbers KM496463, KM496464, KM496465, KM496466, KM496467, and KM496468.

RESULTS

Correlation between serodiagnosis and PCRs for the presence of adhesins. Among 349 *E. coli* isolates, 247 had PCR results consistent with those obtained by serology-based methods for CS31A, F17, and F5, corresponding to a concordance rate of 70.8% (Cohen's kappa, 0.599) (Table 1). The number of false-positive isolates using serology was significantly ($P = 0.003$) higher for CS31A (55/166; 33.1%) than for F17 (13/87; 14.9%) or F5 and F5/F41 (8/38; 21.0%). Among the 74 F17-positive *E. coli* isolates confirmed by PCR, 61 (82.4%) contained one of the 4 F17A variants already reported, the most prevalent being F17Ac (20K) (50%), followed by F17Aa (FY) (18.9%) and F17Ab (8.1%) or F17Ad (111K) (5.4%) (Table 2). Six isolates contained an F17A variant that was untypeable by PCR and that could not be classified from the sequence analysis (see Fig. S3 in the supplemental material). Seven strains contained new F17A combinations, such as F17Aab, F17Aac, or F17Abc. A subcollection of 211 *E. coli* isolates that comprised 111 CS31A-, 74 F17-, and 26 F5- and F41-producing *E. coli* isolates was further studied. The last 26 isolates were either F5 positive by serology and positive for both F5 and F41 by PCR ($n = 24$) or F5- and F41-positive by both methods ($n = 2$). In particular, *E. coli* isolates producing F5 or F41 individually were highly rare and not included in this subcollection. An additional group of 56 diarrheagenic *E. coli* isolates that were negative for CS31A, F17, F5, and F41 by PCR was also included.

TABLE 1 Comparison between serological results obtained by veterinary laboratories and PCR results for the detection of CS31A, F17, F5, and F41 adhesins

PCR results	Serological results (veterinary laboratories)[a]					
	CS31A	F17	F5 + F41	F5	None	Total
CS31A	111	1	0	0	1	113
F17	0	74	0	0	1	75
F5 + F41	0	0	2	24	0	26
F5	0	0	0	4	0	4
None	55	12	1	7	56	131
Total	166	87	3	35	58	349

[a] Number of isolates. The Cohen kappa coefficient calculated with a 95% confidence interval was 0.599; proportion of observed agreement, 70.8%; proportion of expected agreement, 27.2%; proportion of expected agreement minus hazard, 43.6%; maximum agreement not due to hazard, 72.8%.

Phylogenetic analysis. In the subcollection, the proportion of group A was significantly higher among F5/F41-positive isolates than among those expressing other adhesins ($P < 0.00001$). The proportion of group C was also significantly higher among CS31A-positive isolates ($P = 0.003$) (Table 2). In addition, the proportion of B1 was significantly higher among isolates expressing F17 or none of the selected adhesins ($P < 0.00001$). Five out of 6 F17Ab-producing isolates belonged to B1, whereas F17Ac- and F17Aa-producing isolates segregated in similar proportions between the A and B1 groups. Also, F17A combination subtypes, such as F17Aac and F17Abc, and untyped F17A variants were mostly found in the B1 group (Table 2).

Presence of pathovar-related genetic markers among adhesin-producing strains. The overall prevalence of the main *E. coli* pathovars based on the presence of either *stx*, *eae*, *astA*, *sta*, *stb*, *lt*, *bfpA*, or *cnf* genes was 49.8% (133/267), representing 52.3%, 29.7%, 100%, and 48.2% of strains producing CS31A, F17, F5/F41, and no adhesin, respectively (see Table S2 in the supplemental material). All F5/F41-producing *E. coli* isolates were STa positive and belonged to the ETEC pathovar. The presence of the *astA* gene encoding the EAST1 enterotoxin usually related to the enteroaggregative *E. coli* (EAEC) or ETEC pathotype was significantly ($P < 0.001$) higher in CS31A-positive isolates (50.5%) than in those expressing F17 (10.8%), F5/F41 (11.5%), or no adhesin (19.6%). CNF, previously identified in NTEC, was present in 23% (17/74) of the F17-producing isolates, including 5 out of 7 combined F17A subtype-positive isolates and 2 out of 6 untyped F17A variant-expressing isolates. In contrast, CNF was found significantly ($P = 0.0002$) less often in isolates expressing CS31A, F5/F41, or no adhesin. The seven STEC isolates identified did not carry any tested adhesins.

Distribution of virulence genes in selected *E. coli* pathovars. The group of 133 isolates belonging to an *E. coli* pathovar was further analyzed for the presence of additional virulence factors or variants. The 2 *eae*-negative STEC isolates carried stx_{2a} and stx_{2b}, respectively, and the 5 *eae*-positive (*eae* β [4/5] and *eae* θ [1/5]) STEC isolates possessed the stx_{1a} variant, either alone or in combination with stx_{2a} or stx_{2d} (see Table S2 in the supplemental material). The most frequent association was observed between the

TABLE 2 Distribution of phylogenetic groups, pathovar-associated VF genes, and ESBL *E. coli* according to the production of either CS31A, F17, F5/F41, or no adhesin

		Distribution[a]										
			F17									
				F17A variants								
Characteristic	Adhesin	CS31A (n = 111)	Total (n = 74)	F17Aa (n = 14)	F17Ab (n = 6)	F17Ac (n = 37)	F17Ad (n = 4)	F17A combined[c] (n = 7)	NT[e] F17A (n = 6)	F5/F41 (n = 26)	None (n = 56)	P value[b]
Phylogeny	A	12 (10.8)	21 (28.4)	6	0	13	1	0	1	23 (88.5)	13 (23.2)	<0.00001
	B1	9 (8.1)	36 (48.6)	4	5	17	2	4	4	2 (7.7)	26 (46.4)	<0.00001
	B2	4 (3.6)	3 (4.1)	0	0	1	0	1	1	0	2 (3.6)	NA
	C	30 (27.0)	7 (9.5)	1	1	4	0	1	0	1 (3.8)	7 (12.5)	0.003
	D	31 (27.9)	4 (5.4)	2	0	1	0	1	0	0	4 (7.1)	NA
	E	6 (5.4)	1 (1.4)	0	0	0	1	0	0	0	10 (5.4)	NA
	F	15 (13.5)	2 (2.7)	1	0	1	0	0	0	0	1 (1.8)	NA
	NT[e]	4 (3.6)	0	0	0	0	0	0	0	0	0	NA
Pathovar-associated VF genes[d]	STEC											
	stx$_1$	1 (0.9)	0	0	0	0	0	0	0	0	4 (7.1)	NA
	stx$_2$	1 (0.9)	0	0	0	0	0	0	0	0	4 (7.1)	NA
	STEC/EPEC *eae*	1 (0.9)	0	0	0	0	0	0	0	0	8 (14.3)	NA
	EAEC/ETEC *astA*	56 (50.5)	8 (10.8)	5	0	1	0	0	2	3 (11.5)	11 (19.6)	<0.001
	ETEC *sta*	1 (0.9)	0	0	0	0	0	0	0	26 (100)	2 (3.6)	NA
	NTEC *cnf*	3 (2.7)	17 (23.0)	2	5	3	0	5	2	2 (7.7)	7 (12.5)	0.0002
	No VFs	53 (47.7)	22 (39.3)	7 (50.0)	1 (16.7)	33 (89.2)	4 (100.0)	2 (28.6)	3 (50.0)	0	29 (51.8)	0.3
Most prevalent association of VF genes	STEC											
	stx + αH[f]	1	0	0	0	0	0	0	0	0	5	NA
	stx + *eae*	1	0	0	0	0	0	0	0	0	4	NA
	eae + *katP*	0	0	0	0	0	0	0	0	0	5	NA
	eae + *espP*	1	0	0	0	0	0	0	0	0	3	NA
	EAEC/ETEC											
	astA + *katP*	2	3	1	0	0	0	0	2	1	6	NA
	astA + *espP*	9	0	0	0	0	0	0	0	0	0	NA
	astA + *papC*	7	1	0	0	0	0	0	1	0	0	NA
	ETEC											
	sta + *espP*	1	0	0	0	0	0	0	0	17	1	NA
	sta + *astA*	1	0	0	0	0	0	0	0	3	0	NA
	NTEC											
	cnf + *cdtB*	0	10	1	4	1	0	3	1	0	1	NA
	cnf + *katP*	0	9	0	4	3	0	1	1	0	2	NA
	cnf + *astA*	2	3	2	0	0	0	0	1	0	0	NA
Resistance to β-lactam	ESBL											
	CTX-M-1	9 (8.1)	10 (13.5)	2	0	7	1	0	0	2 (7.7)	2 (3.6)	0.2
	CTX-M-2	4 (3.6)	0	0	0	0	0	0	0	0	0	NA
	CTX-M-9	10 (9.0)	1 (1.4)	0	0	0	0	0	1	1 (3.8)	2 (3.6)	NA
	No CTX-M	1 (0.9)	0	0	0	0	0	0	0	0	0	NA
	Total ESBL	24 (22)	11 (14.9)	2	0	7	1	0	1	3 (11.5)	4 (7.1)	0.09

[a] Number of isolates by adhesin group (%).
[b] Comparison of proportions among the different adhesins, CS31A, total F17, F15/F41, and no adhesin. NA, not applicable.
[c] F17Aab (n = 3), F17Abc (n = 1), and F17Aac (n = 3).
[d] *aggR*, *lt*, *stb*, and *bfpA* genes were not detected in the whole collection.
[e] NT, untypeable.
[f] H, hemolysin.

cytolethal distending toxin B (CdtB) and CNF (11/29). The extracellular serine protease (EspP)-encoding genes, which are involved in intestinal colonization and adherence, were found to be significantly linked to F5/F41-positive ETEC ($P = 0.001$), whereas the catalase-peroxidase (KatP) was preferentially identified in F17-positive NTEC isolates (Table 2). The P fimbrial *papC* gene, responsible for adhesion capacity, was mostly detected in isolates carrying both CS31A and *astA* genes. All isolates were negative for *aggR* detection.

Characterization of ESBL production in adhesin-positive isolates. In the subcollection studied, 15.7% were ESBL positive, and CTX-M-1 and -9 were the preponderant subgroups (Table 2). Although a higher proportion of ESBL producers was found among isolates expressing the CS31A adhesin (22%), the differences observed in the occurrence of ESBL production according to the expression of CS31A, F17, or F5/F41 or the absence of adhesin were statistically not significant ($P = 0.09$) (Table 2).

DISCUSSION

In this study, we first investigated the correlation between serology- and PCR-based methods for the detection of adhesins in *E. coli* isolates from diarrheagenic cattle. A 79% to 82.4% correspondence between the two methods was found for fimbrial adhesins, whereas major discrepancies (33%) were observed for CS31A-type antigens. The CS31A antigen did not cross-react with the F17, F5, or F41 antigen. The discrepancy observed between polyclonal serum and PCR for CS31A detection could probably be attributed to the presence of potential new variants of CS31A adhesin that have not yet been identified or to serological cross-reaction between the antigens of other *E. coli* serotypes. On the other hand, the 4 F17A variants already reported were detected by PCR, indicating that the serum used by veterinary laboratories was not selective. However, several F17A isolates positive by serology were not confirmed by PCR, which also suggests the spread of numerous other F17A variants among F17-producing *E. coli* isolates. Altogether, standardization of laboratory methodologies and reagents for the detection and characterization of adhesins in bovine *E. coli* isolates is needed.

In an attempt to evaluate whether the presence of adhesins in bovine-pathogenic *E. coli* would be a relevant indicator of the presence of pathovar-associated VFs, three important findings should be emphasized. First, the highest proportion of VF-positive isolates was observed among *E. coli* isolates producing F5/F41 adhesins. A specific link was also demonstrated between the heat-stable toxins related to the ETEC pathovar and adhesins, with STa being significantly linked to F5/F41 and EAST1 to CS31A, whereas the NTEC pathovar was associated with the F17 adhesin (especially F17Ab and new F17A combinations or untypeable F17A variants). Finally an association between phylogroups and adhesin types was observed. The specific link observed between toxins, phylogroups, and adhesins strongly suggests a correlation between certain adhesion factors of *E. coli*, colonization, and infection mechanisms.

Here, we also demonstrated that the STEC isolates identified in this study did not produce any adhesin tested; therefore, the use of CS31A, F17, F5, or F41 identification-based methods failed to isolate STEC. In addition, the enteropathogenic *E. coli* (EPEC) pathovar was not identified in the whole collection. The low proportion of STEC or EPEC isolates among *E. coli* isolates from diseased cattle confirmed previous observations (20). In contrast, compared to the STEC, NTEC, and ETEC pathotypes, EAEC isolates are not yet well characterized, and their virulence genes are heterogeneous among isolates. Although the EAEC-related genetic marker *aggR* was not found in our collection, which suggests the absence of EAEC in cattle, more investigations would be useful to assess the significance of EAST1 in calf enteritis, especially for EAEC- or ETEC-associated diarrhea.

Also, more than half of the *E. coli* isolates isolated from diarrheic calves did not present any of the tested VFs. It may be argued that VFs responsible for calf diarrhea still remain undefined, probably because most attention has been focused on VFs associated with human disease. Another hypothesis could be that the pathogenic isolates were underrepresented within samples and therefore were not detected. Altogether, whether these isolates were actually the cause of the intestinal infections remains an open question, reinforcing the urgent need for more accurate diagnostic tools to clarify the causes of enteric diseases in young cattle.

Finally, as antimicrobials have become a major driving force in the evolution of *E. coli* populations in livestock, we investigated a possible correlation between adhesins, VFs, and ESBL gene carriage. The finding of ESBL-producing STEC strains from animal sources is rare (21). In this study, STEC isolates did not produce ESBLs, unlike the other *E. coli* pathotypes. Similarly, the prevalence of adhesin genes was higher in non-ESBL-producing isolates (22). This important result supports the hypothesis that the antimicrobial resistance and virulence of *E. coli* do not necessarily follow similar selective pathways, in spite of the high prevalence of ESBL producers observed in the whole collection of strains.

In conclusion, the conventional identification of F5/F41, F17, or CS31A adhesins in clinical *E. coli* isolates is a possible indicator for the detection of specific pathovars, such as ETEC or NTEC, or of EAST1-producing isolates. However, it failed to detect the presence of STEC, and a virulence gene identification-based method is surely more suitable. Although no significant correlation was found between *E. coli* pathotypes or adhesins and the occurrence of ESBL, ESBL production was found in association with CNF, EAST1, and STa toxins in some isolates in this study, suggesting that ESBL genes would probably be able to propagate to strains pathogenic to humans, as well.

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