2. Etude #2 :

Recherche d'adhésines

Cette étude est basée sur les deux manuscrits suivants :

- Presence in bovine enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* of genes encoding for putative adhesins of human EHEC strains.

Ioan Mihai Szalo, Frédéric Goffaux, Vinciane Pirson, Denis Piérard, Hywel Ball, Jacques Mainil

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- Identification of sequences homologues to genes encoding for AFA adhesins family, in bovine O8 and O20 verotoxigenic *Escherichia coli*.

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2.1. Préambule

Rien n'était connu en 2000 sur les adhésines spécifiques impliquées dans le processus d'adhérence initiale des souches EPEC, EHEC et VTEC bovines. Par contre, des adhésines spécifiques ou des facteurs impliqués dans le processus d'adhérence initiale étaient décrits pour les souches EPEC d'origine humaines et de lapins, ainsi que pour les souches VTEC porcines de la maladie de l'œdème (Giron *et al.*, 1991 ; Imberechts et al., 1992 ; Milon *et al.*, 1999). Nous nous sommes proposé donc de rechercher, par des outils génétiques, la présence de séquences d'ADN homologues aux opérons qui codent pour les fimbriae de type I, II, IV et pour les adhésines afimbriaires de la famille Afa parmi les souches EPEC et EHEC (article #4) et les souches VTEC (article #5) bovines de notre collection. Plus précissement, nous avons recherché dans notre collection de souches EPEC et EHEC bovines la présence de gènes codant (i) pour des adhésines potentielles des souches EHEC humaines, telle que LifA, Iha, LpfA, BfpA et (ii) pour des adhésines fimbriaires et afimbriaires présentes chez d'autres catégories de souches d'*E. coli* pathogènes pour le bovin, incluant Afa8-D/E, CS31A et F17A ; et parmi notre collection des souches VTEC d'origine bovine de sérogroupe O 8 et O20 des gènes intervenant dans la biosynthèse d'adhésines de la famille Afa (Afa III, Afa VII, Afa VIII et F1845).

Les résultats obtenus ont permis la mise en évidence une association entre le gène *lpfA* et les souches EPEC et EHEC bovines ainsi que les souches EHEC humaines appartenant au sérogroupe O145 et O157 ainsi qu'entre le gène ClpE (codant pour la protéine chaperon du fimbria CS31A) et les souches EPEC bovines de sérogroupe O26. De plus, la recherche des gènes intervenant dans la biosynthèse d'adhésines de la famille Afa (Afa III, Afa VII, Afa VIII and F1845) dans la collection des souches VTEC d'origine bovine de sérogroupe O8 et O20 a permis la mise en évidence de deux souches VTEC de sérogroupe O8 qui possèdent les gènes *afa-8D/afa-8E* et qui expriment l'adhésine Afa 8E.

2.2. Article #4

Presence in bovine enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* of genes encoding for putative adhesins of human EHEC strains.

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Presence in bovine enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* of genes encoding for putative adhesins of human EHEC strains

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Abstract

Enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC) infections are characterised by the formation of attaching and effacing lesions on intestinal epithelial cells. The first step of EPEC and EHEC pathogenesis involves the initial adherence of the bacterium to the intestinal epithelium. A collection of bovine EPEC and EHEC strains belonging to different serogroups was tested by colony blot hybridization with gene probes for putative adhesins (BFPA, LPFA, IHA, LIFA) of human EPEC and EHEC, and also for fimbrial and afimbrial adhesins (AFA8, F17, Cs31A) of bovine necrotoxigenic *E. coli* (NTEC). In the bovine EPEC and EHEC strains tested, sequences homologous to *lifA*, *ihA*, and *lpfA* genes were detected, sometimes in association with particular serogroups. Bovine O26 EPEC also possessed a sequence homologous to a gene of the *clp* operon, coding for the CS31A adhesin, associated with bovine NTEC. Overall results showed that different genes encoding for putative adhesins of human EHEC strains are present in bovine EPEC and EHEC strains, but not one of them is present in all strains.

Keywords: Bovine *Escherichia coli*; EPEC; EHEC; Adhesin

1. Introduction

Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* are implicated in diarrhoea and/or dysentery in human and ruminants [3,5,21,24]. EPEC and EHEC are distinct from other pathogenic *E. coli* because of the production of a histopathological lesion on gut enterocytes, called the "attaching and effacing" (AE) lesion. The AE lesion is characterized by the intimate attachment of bacteria to the enterocyte and by the localized effacement of microvilli [20].

The first step in EPEC and EHEC pathogenesis, as for many enteropathogenic bacteria, is the colonisation of the intestinal mucosal surface to prevent their elimination by peristalsis.

EPEC form dense microcolonies on the surface of HEp-2 cells in a pattern known as localized adherence (LA) [28]. The principal factor responsible for the LA phenotype is a plasmid-encoded bundle forming pilus (BFP), member of the type IV fimbria family [9]. An EPEC strain mutated in the *bfpA* gene, encoding for the major structural subunit of BFP, failed to form aggregates and does not display localized adherence [7]. But in a human intestinal organ culture model, BFP appears to mediate bacterium–bacterium interactions rather than direct binding to epithelial cells [13].

Although EHEC strains contain large plasmids similar to those of EPEC and are able to adhere to cultured epithelial cells, they lack the genes required for the synthesis of BFP [30]. The mechanisms underlying the adherence of EHEC strains to epithelial cells is still poorly understood, but some putative adhesins have been recently described. A chromosomal region containing six open reading frames (*lpfABCC'DE*), which are closely related to the long polar fimbria operon of *Salmonella* serotype *Typhimurium*, was

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recently identified in an EHEC O157:H7 [32]. An *lpfA* mutant shows a 30% reduction in adherence to tissue culture cells and is unable to form microcolonies. Furthermore, a chromosomal gene coding for a protein similar to the IrgA (iron-regulated gene A) protein of *Vibrio cholerae,* and conferring the capacity to adhere to epithelial cells in a diffuse pattern, was recently identified in *E. coli* O157:H7 [31]. The product of this gene was called Iha (IrgA homologue adhesion). On the other hand one transposon-generated mutant from an EHEC O111:H- is deficient for the adhesion to cultured Chinese hamster ovary (CHO) cells [22]. In this mutant, the transposon is inserted in the *efa1* gene, which is 100% identical to the *lifA* gene encoding a large toxin of EPEC that inhibits lymphocyte activation [14].

The initial adhesion factor of bovine EPEC and EHEC, if any, also awaits identification, although positive adherence properties to culture cells were described [12,33,34]. The purpose of this study was to test the presence, in bovine EPEC and EHEC strains, of genes coding for putative adhesins found in human EHEC (Lpf, LifA, Iha), and for fimbrial and afimbrial adhesins (Afa8, F17 and Cs31A), recently associated with bovine necrotoxigenic *E. coli* (NTEC) [1,8,17].

2. Materials and methods

2.1. E. coli *isolates*

Thirty-eight EHEC of different serogroups (O5, O26, O103, O111 and O118), 10 EPEC of serogroup O26 isolated from faeces or intestine of young calves [19,26], and 18 O157 and 2 O145 EHEC isolated at the slaughterhouse from beef carcasses, were studied. Fifty-six EHEC isolated from humans suffering from haemorrhagic colitis or haemolytic uremic syndrome, and belonging to the same serogroups as bovine strains, were also included in this study.

2.2. Gene probe derivation and colony hybridization

The DNA colony hybridization was performed on paper filters (Whatman 541, Belgolabo, Overijse, Belgium) at 65 °C, as previously described [18].

The specific probes were derived by PCR from genes *afa8-D* (AFA8-D probe), *afa8-E* (AFA8-E probe), *f17A* (F17A probe), *f17G* (subtype II; F17GII probe), *bfpA* (BFPA probe), *lpfA* (LPFA probe), *clpE* (CS31-E probe), *clpG* (CS31-G probe), *lifA* (LIFA probe) and *iha* (IHA probe) (Table 1). The positive controls were strains 239KH89, carrying the *afa8* operon, 25KH9 and S5, carrying the *f17* operons, E2348/69 carrying the *bfp* operon and the *lifA* gene, ATCC43888, carrying the *lpf* operon and the *iha* gene, and 31A, carrying the *clp* operon [1,2,11,15]. The non-pathogenic human strain HS (O9:H4) [23] was used as a negative control.

The different DNA probe fragments were purified by the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and labelled with α^{32}P-dCTP by random priming using the dCTP-labelling beads (Ready-to-go, Pharmacia, Uppsala, Sweden). Labelled DNA probes were purified with Microcon-YM30 spin columns (Millipore, St Quentin Yvelines, France).

Table 1
Sequences of primers used in this study

Gene (adhesin)	Primers	Annealing temp. (°C)	Length of the PCR product (bp)	Reference
afa8-D (Afa-VIII)	5′-GTTGAACTGAGTCTTAATACCAGTG-3′ 5′-TGAGCATTCTCCGCTAACTGATAAT-3′	65	354	[15]
afa8-E (Afa-VIII)	5′-CTAACTTGCCATGCTGTGACAGTA-3′ 5′-TTATCCCCTGCGTAGTTGTGAATC-3′	65	302	[15]
f17A (F17)	5′-GCAGAAAATTCAATTTATCCTTGG-3′ 5′-CTGATAAGCGATGGTGTAATTAAC-3′	55	537	[2]
f17G-II (F17)	5′-CGTGGGAAATTATCTATCAACG-3′ 5′-TGTTGATATTCCGTTAACCGTAC-3′	55	615	[2]
clpE (Cs31A)	5′-GGTCAGGCCTGGGTGGACAATATC-3′ 5′-GCGATAGAACAGTTTCAGCTTCGT-3′	58	240	This study
clpG (Cs31A)	5′-GGGCGCTCTCTCCTTCAAC-3′ 5′-CGCCCTAATTGCTGGCGAC-3′	55	403	[1]
bfpA (BFP)	5′-AATGGTGCTTGCGCTTGCTGC-3′ 5′-GCCGCTTTATCCAACCTGGTA-3′	56	326	[11]
lpfA (Lpf)	5′-CTGCGCATTGCCGTAAC-3′ 5′-ATTTACAGGCGAGATCGTG-3′	54	412	This study
lifA (LifA)	5′-CCCTGATAAGCACGCCTAA-3′ 5′-CCACACTCATGCCGAGGTC-3′	57	894	This study
iha (IrgA)	5′-CAAATGGCTCTCTTCCGTCAATGC-3′ 5′-CAGGTCGGGGTTACCAAGT-3′	59	925	This study

2.3. Polymerase chain reactions

PCR amplifications were performed in a thermocycler PCR Sprint (Labsystems, Bruxelles, Belgium) using Taq DNA polymerase (Roche, Germany), as described previously [6]. The primer sequences (Gibco BRL, Merelbeke, Belgium) and PCR product sizes are shown in Table 1. Positive and negative controls were as for colony hybridization.

2.4. Reverse transcription-PCR

Overnight broth cultures were diluted 1/50 in Penassay broth (Oxoid, Drongen, Belgium) and grown until the OD_{600} was 0.6–1.0. RNA was extracted using the SV total RNA isolation system kit (Promega, Leiden, The Netherlands) following the recommendations of the manufacturer. For RT-PCR, 0.5 µl of primer clpE-upper (50 pmol) mixed with 10 µl of DEPC-treated water containing RNA was incubated at 68 °C for 10 min and then at 4 °C for 15 min. Each tube received 1 µl of a solution containing each nucleotide triphosphate (10 mmol), 0.1 µl of AMV enzyme (1 U) (Promega), 5 µl of AMV 5X buffer, and DEPC-treated water was added to obtain a final volume of 25 µl. The reaction mixture was incubated at 42 °C for 45 min, 94 °C for 10 min, and finally 4 °C for 15 min. Five microlitres of cDNA were used to perform PCR with primers clpE-upper and clpE-lower as described previously [6].

2.5. DNA sequencing

DNA fragments produced by PCR were purified using the QiaQuick PCR purification kit (Qiagen). Sequencing of the two DNA strands was performed by the dideoxynucleotide chain termination method [27]. Automatic reading was performed using an ALF DNA sequencer (Pharmacia) and Cy-5 fluorescent labeled primers from Pharmacia. Sequence comparisons were performed using GeneWorks 2.4 (IntelliGenetics, Mountain View, CA).

3. Results

3.1. Detection of DNA sequences homologous to genes coding for human EHEC putative adhesins (Table 2)

All bovine and human EHEC strains belonging to the O145 (9 strains) and O157 (32 strains) serogroups were positive with the LPFA probe; 46 of 48 bovine EPEC and EHEC strains and 34 of 35 human EHEC strains, belonging to serogroups other than O157 and O145 were positive with the LIFA probe. Moreover, a majority of

Table 2
Results of colony blot with the different DNA probes

Strain	AFA8-D/E	F17A/GII	CS31-E/G	BFPA	LPFA	LIFA	IHA
Bovine							
O26							
EPEC (12)	–/–	–/–	+ (10)/–	–	–	+ (12)	+ (1)
EHEC (12)	–/–	–/–	–/–	–	–	+ (12)	+ (12)
O118							
EHEC (7)	–/–	–/–	–/–	–	–	+ (7)	+ (7)
O111							
EHEC (6)	–/–	–/–	–/–	–	–	+ (6)	+ (6)
O103							
EHEC (7)	–/–	–/–	–/–	–	–	+ (5)	+ (3)
O5							
EHEC (4)	–/–	–/–	–/–	–	–	+ (4)	+ (1)
O145							
EHEC (2)	–/–	–/–	–/–	–	+ (2)	–	+ (1)
O157							
EHEC (18)	–/–	–/–	–/–	–	+ (18)	–	+ (18)
Human							
O26							
EHEC (9)	–/–	–/–	–/–	–	–	+ (9)	+ (9)
O111							
EHEC (6)	–/–	–/–	–/–	–	–	+ (6)	+ (6)
O145							
EHEC (7)	–/–	–/–	–/–	–	+ (7)	–	+ (7)
O103							
EHEC (16)	–/–	–/–	–/–	–	–	+ (15)	+ (3)
O118							
EHEC (4)	–/–	–/–	–/–	–	–	+ (4)	+ (4)
O157							
EHEC (14)	–/–	–/–	–/–	–	+ (14)	–	+ (11)

Number of strains are in brackets: +, positive result; –, negative result.

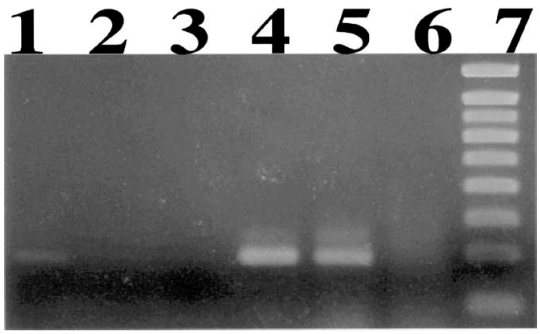

Fig. 1. Results of RT-PCR on 3 bovine O26 EPEC strains. Lane 1: 631KH91 (cDNA); lane 2: 352KH91 (cDNA); lane 3: 334KH91 (cDNA); lane 4: 31A (cDNA); lane 5: 31A (DNA); lane 6: HS (DNA); lane 7: smart ladder small fragments (Eurogentec, Seraing, Belgium).

bovine and human EHEC strains belonging to O157, O145 and other serogroups, but only one out of 12 bovine EPEC of serogroup O26, also tested positive with the IHA probe. On the other hand, all strains tested negative with the BFPA probe.

3.2. Detection of DNA sequences homologous to genes encoding for bovine NTEC adhesins (Table 2)

Ten bovine O26 EPEC out of 12 were positive with the CS31-E probe, but all other strains tested negative with that probe. All strains also tested negative with AFA, F17 and CS31-G probes.

Three strains (334KH91, 352KH91, and 631KH91) were chosen for further studies. PCR amplification products obtained from these 3 strains with clpE primers (240 bp) were sequenced and analysed. The 3 sequences showed 100% identity together and comparison of these sequences with the *clpE* gene showed 89% identity. We also tested these 3 bovine EPEC strains by RT-PCR to determine if the *clpE*-like gene was transcribed. Results obtained were negative with the 3 bovine O26 EPEC and positive with the reference strain 31A (Fig. 1).

4. Discussion

EPEC and EHEC strains share the capacity to produce a lesion characterized by the intimate attachment of bacteria to the enterocytes and by the effacement of microvilli (AE lesion). While this step in EPEC and EHEC pathogenesis was recognized years ago, the actual adhesin(s) involved in the primary colonisation of the intestinal tract still awaits formal identification.

While type IV BFP pili play a role in this colonisation step by human EPEC strains [29], bovine EPEC and EHEC, as human EHEC strains tested in this study, do not possess any sequence homologous to the *bfpA* gene. Nevertheless, all bovine and human EHEC strains belonging to the O145 and O157 serogroups possess a sequence homologous to the *lpfA* gene, also coding for type IV pili. This result is even more interesting since O157 and O145 bovine and human EHEC strains belong to the same LEE (locus of enterocyte effacement) pathotype (*eaeγ-tirγ-espγ*), based on the typing of genes necessary for the production of the AE lesion [4,10, unpublished data]. Thus, the presence of the *lpfA* gene, or a related sequence, is not only associated with these 2 serogroups but also with a particular pathotype. Nevertheless, the *lpfA* and *eae-tir-esp* genes are not located on the same pathogenicity island [25]. Since beef and dairy products are considered as major sources of O157 infections in human, it is also interesting to note that this gene is present in bovine and as well as human O157 strains.

In contrast, almost all bovine and human EPEC and EHEC of other serogroups possess a sequence homologous to the *lifA* gene. These results are in agreement with previous studies showing that all human EHEC and EPEC strains tested except O157:H7 EHEC possess a sequence homologous to the *lifA* gene [14,22]. The role of the LifA toxin in adherence is still controversial since a *lifA* mutant of the human EPEC strain E2348/69 was still able to produce the localized adherence phenotype on HEp-2 cells [14], while an *efa1* (100% identical to *lifA*) mutant of the human EHEC strain E45035 (O111:H-) was not able to do so on CHO cells [22].

Though the *iha* gene seems to be present primarily in EHEC rather than in EPEC strains, more strains of the latter should be tested before further discussing these results. The results are also variable amongst EHEC according to the serogroup but in a different way then with the *lpfA* and *lifA* genes. It should be noted that the product of the *iha* gene confers diffuse adherence rather than localized adherence in *E. coli* O157:H7 [31].

Three categories of pathogenic *E. coli* are responsible for diarrhea in calves: EPEC/EHEC, NTEC, and enterotoxigenic *E. coli* (ETEC) [16]. Interestingly, a sequence homologous to the *clpE* gene, coding for the chaperone protein of the Cs31A fimbriae in bovine NTEC [1], was detected in most bovine EPEC strains of the O26 serogroup. Bovine and human EHEC of the same serogroup do not possess such a sequence. Sequencing of the amplification products shows a high percentage of identity with the *clpE* gene of the bovine reference strain 31A, but the result of RT-PCR indicates that this gene is not expressed in bovine EPEC. Furthermore, these strains do not harbour the entire *clp* operon since the *clpG* gene, coding for the major structural subunit of the Cs31A fimbriae, was not detected by colony blot hybridization.

In conclusion, this study showed that (i) bovine EPEC and EHEC possess genes encoding for putative adhesins present in human EHEC strains, (ii) DNA sequences related to genes encoding for different adhesins are present in bovine EPEC and EHEC strains but none is present in all strains, (iii) O145 and O157 EHEC strains with a specific pathotype may form a class apart, and (iv) it is not possible

to differentiate bovine and human EHEC strains based on the presence of genes encoding for adhesins possibly responsible for the initial adherence phenotype. The basis of their host specificity, if any, shall be looked for in other properties and by other methods. Further studies on the exact role of these putative adhesins should compare bovine wild type strains and their allelic mutants for their adherence properties on cell cultures, intestinal explants and finally animal models.

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