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Examination of *Escherichia coli* from poultry for selected adhesin genes important in disease caused by mammalian pathogenic *E. coli*

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Abstract

A collection of 1601 extraintestinal and intestinal *Escherichia coli* isolated from chickens, turkeys and ducks, in Belgium, France and Spain, was hybridised with gene probes specific for fimbrial and afimbrial adhesins (F17, F18, S ⟨Sfa/F1C⟩, Bfp, Afa, Cs31A, Intimin ⟨Eae⟩, Aida-1) of intestinal, urinary and invasive *E. coli* of mammals and with a probe specific for the P (Pap/Prs) fimbrial adhesin of urinary and invasive *E. coli* of mammals and birds. Three hundred and eighty-three strains (23.9%) were P-positive, 76 strains (4.8%) were Afa-positive, 75 strains (4.7%) were F17-positive, 67 strains (4.2%) were S-positive, 23 (1.4%) were Intimin-positive, and all were F18-, Cs31A-, Aida1- and Bfp-negative. The 75 F17-positive strains harboured different major subunit A-encoding gene variants, but the *f17*Ac variant was the most frequent (52 strains, 69.3%) and seven strains (9.3%) were not typeable. The *f17*G gene variant coding for the GII adhesin was the most frequent (56 strains, 75.0%), whereas the *f17*GI gene variant was present in four strains (5%) and 15 strains (20.0%) were not typeable. All Afa-positive strains harboured the *afa*-8 variant. The 23 Intimin-positive *E. coli* tested positive for the β-variant (16 strains; 69.6%) or for the γ-variant (seven strains; 30.4%) of the *eae* gene. Chicken and turkey *E. coli* were more frequently probe-positive (43.6 and 43.1%, respectively) than duck *E. coli* (31.5%) and extraintestinal *E. coli*

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were also more frequently probe-positive (48.4%) than intestinal strains (18.5%). Different combinations of probe positive hybridisation results were observed in 72 of the 540 probe-positive *E. coli* (13.3%). The most frequent combinations were between AfaE-8 and F17 probes (47 strains; 8.7%) and between P and S probes (13 strains; 2.4%). Although *f17*- and *afa*-8-related DNA sequences can be plasmid-located in mammalian *E. coli*, they were not in avian *E. coli*. Besides the P fimbrial adhesins, F17 and S fimbrial and Afa-VIII and Intimin afimbrial adhesins may thus represent colonisation factors of avian pathogenic *E. coli*.

Keywords: *Escherichia coli*; Adhesins; Gene probes; PCR; Chicken

1. Introduction

Escherichia coli are commensal bacteria of the intestine of animals, but some strains are associated with intestinal, respiratory, urinary or invasive infections. Avian pathogenic *E. coli* (APEC) are often associated with other pathogens such as mycoplasma and viruses or with environmental hazards like dust and high concentration of ammonium in the air (Oyetunde et al., 1978; Gross, 1994). The most frequent pathologies in poultry (chickens, turkeys) associated with *E. coli* are systemic infections with lesions of aerosacculitis, pericarditis, perihepatitis, peritonitis and salpingitis. The main route of entry is the respiratory tract after the inhalation of faeces-contaminated dust. The intestine is the most important reservoir of APEC. After an initial multiplication in the upper respiratory tract, the bacteria colonise the air sacs and the lungs. In a second stage of infection, the bacteria reach the blood and colonise the internal organs such as heart, spleen and liver (Dho-Moulin and Fairbrother, 1999).

APEC are restricted to a few important O-serogroups, with 15–61% of the total number of isolates belonging to serogroups O1, O2 and O78, but their specific virulence factors have not been completely identified (Babai et al., 1997; Blanco et al., 1998). The colonisation of the respiratory tract is most probably mediated by adhesins, such as the P fimbriae which are more frequently expressed by septicaemic APEC than by *E. coli* isolated from healthy chickens (Dozois et al., 1992). Type 1 fimbriae are no longer considered important virulence factors. The production of a capsule and of an aerobactin iron-sequestering system, and resistance to the bactericidal activity of complement, represent virulence properties which help the APEC to survive in the blood stream (Ellis et al., 1988; Lafont et al., 1987).

The association between *E. coli* and enteritis in poultry is controversial. Indeed, some enteric virulence properties have been described in a very few isolates, such as production of classical heat-stable and heat-labile enterotoxins (STa and LT), of a verocytotoxin called VT2y (Parreira et al., 1998), or of the attaching/effacing lesion, but no colonisation factor typical of intestinal APEC has yet been reported (Dho-Moulin and Fairbrother, 1999).

The purpose of this study on a large collection of extraintestinal and intestinal *E. coli* isolated from chickens, turkeys and ducks, in Belgium, France and Spain, was to determine: (1) the prevalence of DNA sequences coding for fimbrial and afimbrial adhesins of intestinal, urinary and invasive *E. coli* of mammals (F17, F18, S⟨Sfa/F1C⟩, Bfp, Afa, Cs31A, Intimin⟨Eae⟩, Aida1); (2) the association of these sequences with those coding

for P (Pap/Prs) fimbriae; their localisation on plasmids or on the chromosome and, the subtyping of the adhesin-encoding sequences detected.

2. Material and methods

2.1. Collection of strains

A collection of 1601 avian *E. coli* strains isolated from intestine, heart, spleen, liver, air sacs (65% invasive strains; 13.5% intestinal strains; 21.5% strains with no data) of poultry was obtained from three countries (Spain, France and Belgium). Complete case history was available for 971 strains.

2.2. Gene probe derivation and colony hybridisation

The F17, F18, Aida1, Bfp and Cs31A probes were derived by restriction of recombinant plasmids and the Afa, Pap, Sfa, Eae, F17GI, F17GII and AfaE-8 probes, as PCR products.

The F17, F18, Afa, Pap, Sfa and Eae probes detect all known *f17*-, *f18*-, *afa*-, *pap*/*prs*-, *sfa*/*foc*-, and *eae*-related genes or gene clusters, respectively (Smyth et al., 1994; Bertin et al., 1996a; Donnenberg and Welch, 1996; Johnson, 1997; Mainil et al., 1997, 2000; Adu-Bobie et al., 1998; Le Bouguénec and Bertin, 1999; Nagy and Fekete, 1999; China et al., 1999b; Gérardin et al., 2000; Oswald et al., 2000). The positive controls were strains 25KH9 (F17+) (Lintermans et al., 1988), 107/86 (F18+) (Imberechts et al., 1994), A30 (Afa+) (Le Bouguénec et al., 1992), J96 (Pap+Sfa+) (Blum et al., 1995) and enteropathogenic (EPEC) strain E2348/69 (Eae+) (Jerse et al., 1990).

The F17GI, F17GII, Aida1, Bfp, Cs31A and AfaE-8 probes are specific for their respective genes or gene clusters of origin (Benz and Schmidt, 1989; Martin et al., 1991; Giron et al., 1993; Bertin et al., 1996b; Lalioui et al., 1999). The positive controls were strains 25KH9 (F17GI+) (Bertin et al., 1996b), S5 (F17GII+) (Bertin et al., 1996b), 2787 (Aida1+) (Benz and Schmidt, 1989), E2348/69 (Bfp+) (Jerse et al., 1990), 31A (Cs31A+) (Martin et al., 1991) and necrotoxigenic (NTEC) strain 239KH89 (AfaE-8+) (Mainil et al., 1997).

The different DNA probe fragments were purified by the Geneclean II kit (Bio 101, Buena Vista, CA) or 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, Beverly, MA).

The DNA colony hybridisation was performed on paper filters (Whatman 541, Belgolabo, Overijse, Belgium) at 65 °C (Mainil et al., 1997). The negative control for all probes was the HS strain isolated from the faeces of an asymptomatic human (O'Brien et al., 1982).

2.3. Family PCR reaction

The F17 probe-positive strains were confirmed by a family PCR which detects all the four variants of the *f17*A gene coding for the major subunit (Bertin et al., 1996b). The

control strains were 25KH9 (*f17*Aa variant) (Lintermans et al., 1988), S5 (*f17*Ab variant) (El Mazouari et al., 1994), 31A (*f17*Ac variant) (Bertin et al., 1996a), and 111KH86 (*f17*Ad variant) (Bertels et al., 1989).

2.4. Subtyping of F17-, Afa- and Eae-positive strains

F17-, Afa- and Eae-positive strains were further studied to identify the gene variants coding for these adhesins.

F17-positive strains were subtyped by multiplex PCR assays for the a, b, c, and d variants of the *f17*A gene, which codes for the major fimbrial subunit and for the two *f17*G gene variants (I, II) which codes for the fimbrial adhesin (Bertin et al., 1996b). The *f17*G gene subtyping was completed using the F17GI and F17GII gene probes derived by PCR from the *f17*GI and *f17*GII gene variants. The positive controls were strains 25KH9 (*f17*Aa and *f17*GI variants), S5 (*f17*Ab and *f17*GII variants), 31A (*f17*Ac variant), and 111KH86 (*f17*Ad variant).

Afa-positive strains were subtyped using gene probe and PCR assays for the *afa*D-8 and *afa*E-8 genes, wich codes for an invasin and an adhesin, respectively (Lalioui et al., 1999), since previous results (Gérardin et al., 2000) identified the *afa* variant present in a few avian *E. coli* as *afa*-8. The positive control was the NTEC strain 239KH89 (Mainil et al., 1997).

Eae-positive strains were subtyped using a multiplex PCR assay for the α-, β- and γ-variants of the *eae* gene. Positive controls were the EPEC strain E2348/69 for subtype α, the EPEC strain RDEC-1 for subtype β and the O157:H7 enterohaemorrhagic (EHEC) strain ATCC43888 for subtype γ (China et al., 1999a).

2.5. Plasmid DNA hybridisation

Since *f17*- and *afa-8*-related DNA sequences can be plasmid-located in mammalian *E. coli* (Mainil et al., 1997, 1999; Le Bouguénec and Bertin, 1999; Gérardin et al., 2000), plasmid DNA was extracted from the F17- and Afa-positive strains according to the method described by Kado and Liu as modified by Broes et al. (1988). The plasmid bands were separated by electrophoresis (20 h at 40 V) in 0.5% agarose gel. The size ladders were the plasmids from *E. coli* strain 39R681: 98, 42, 23.9 and 4.6 Mda (Mainil et al., 1997). Gels were treated and hybridised, as described previously (Broes et al., 1988), with the F17 and AfaE-8 probes. The plasmid positive controls were extracted from the NTEC strains 1648S89 and 1649S89 for the F17 and AfaE-8 probes, respectively (Mainil et al., 2000; Gérardin et al., 2000).

3. Results

3.1. Prevalence of adhesin-encoding genes

Two hundred and thirty-five (14.7%) of the 1601 *E. coli* of the collection tested positive with the F17, Afa, Sfa and/or Eae probes, and none with the F18, Cs31A, Aidal and Bfp probes.

Table 1
Origin (host, isolation site) of 408 probe-positive avian *E. coli* with case history

Probes	Host/site									Total (971)		
	Chicken (528)[a]			Turkey (332)			Duck (111)			E (763)	I (208)	%
	E[b] (380)[a]	I[c] (148)	%	E (272)	I (60)	%	E (111)	I (0)	%			
F17	28[d]	0	5.3[e]	23	1	7.2	8	0	8.0	59	1	6.2
Afa	15	8	4.3	21	0	6.3	9	0	8.1	45	8	5.5
Sfa	22	1	4.3	17	1	5.4	2	0	1.8	41	2	4.4
P	139	23	30.7	102	2	31.3	23	0	20.7	264	25	29.8
Eae	6	4	1.8	2	0	<1	1	0	<1	9	4	1.3
Total	194	36	43.6	140	3	43.1	35	0	31.5	369	39	42.0

[a] Number of strains tested.
[b] Extraintestinal.
[c] Intestinal.
[d] Number of probe-positive strains.
[e] Percentage of total number of strains tested (footnote a) that were probe-positive.

Sixty-four *E. coli* (4.0%) gave a strong positive signal and 57 (3.6%) a weak positive signal with the F17 probe compared to the positive control strain. The F17-family PCR was subsequently applied on these 121 *E. coli* and gave positive results with 75 (4.7%) of them. Only these 75 strains were further studied. Seventy-five strains (4.7%) of the collection tested positive with the Afa probe, 67 (4.2%) with the Sfa probe and 23 (1.4%) with the Eae probe. Finally, 383 strains (23.9%) were positive with the Pap probe.

Complete case histories were available for 408 probe-positive strains (Table 1). The strains isolated from chickens (528) and turkeys (332) were more frequently probe-positive (43.6 and 43.1%, respectively) than those isolated from ducks (111, 31.5% probe-positive), as were the extraintestinal strains (763, 48.4% probe-positive) compared to the intestinal strains (208, 18.5% probe-positive) (Table 1). Among groups of probe-positive strains, the majority also originated from extraintestinal organs: between 70% (Eae probe) and more than 99% (P probe) (Table 1).

3.2. Subtyping of F17-, Afa- and Eae-positive E. coli

The 75 F17-positive strains were subtyped using multiplex PCR for the a, b, c, and d variants of the *A* gene: 52 strains (69.3%) were *f17*Ac-positive, six strains (8.0%) were *f17*Ac-and *f17*Ad-positive, four strains (5.3%) were *f17*Aa-positive, three strains (4.0%) were *f17*Aa- and *f17*Ac-positive, three strains (4.0%) were *f17*Ad-positive and seven strains (9.3%) were not typeable. No positive result was obtained with the PCR for the F17b variant.

The PCR subtyping results of the *G* gene were as follows: 36 strains (48.0%) were *f17*GII and none *f17*GI (Table 2). Of the 39 strains (52.0%) with no results by PCR, four (5.3%) were positive with the F17GI probe and 20 (26.7%) with the F17GII probe. Fifteen F17-positive strains (20.0%) were thus negative by PCR and by hybridisation for the *f17*G gene (Table 2).

Table 2
Subtyping results of the *f17*A and *f17*G genes of 75 F17 family PCR-positive *E. coli*

*f17*A gene subtypes (No. strains)	*f17*G gene subtyping						
	*f17*GI		*f17*GII		No results		
	PCR	Probe	PCR	Probe	PCR	Probe	PCR + probe
*f17*Aa (4)	0	4	0	0	4	0	0
*f17*Ac (52)	0	0	29	41	14	2	9
*f17*Ad (3)	0	0	1	1	0	0	2
*f17*Aa/*f17*Ad (3)	0	0	0	2	2	0	1
*f17*Ac/*f17*Ad (6)	0	0	1	5	4	0	1
No results (7)	0	0	5	5	0	0	2
Total (75)	0	4	36	54	24	2	15

All 75 Afa-positive strains were confirmed as harbouring an *afa*E-8 gene and an additional one was detected by colony hybridisation with the AfaE-8 specific probe. All 76 AfaE-8 probe-positive strains also tested positive with the PCR for the *afa*D-8 and *afa*E-8 genes.

The 23 Eae-positive *E. coli* tested positive by the PCR for the β-variant (16 strains; 69.6%) or for the γ-variant (seven strains; 30.4%).

3.3. Association between positive probe hybridisation results

Different combinations of positive probe hybridisation results were observed (Table 3) in 72 of the 540 probe-positive *E. coli* (13.3%). The most frequent combination was

Table 3
Combination of probes on the 540 *E. coli* positive strains by colony hybridisation

Hybridisation results[a]	No. of positive strains (total of 540 strains)	Percentage of positive strains
P	351	65
S	52	9.6
AfaE-8[b]/F17	37	6.9
AfaE-8	24	4.4
F17	22	4.1
Eae	19	3.5
P/S	11	2.0
P/F17/AfaE-8	8	1.5
P/F17	4	<1
AfaE-8/P	4	<1
Eae/P	3	<1
P/S/F17	2	<1
F17/AfaE-8/S	2	<1
AfaE-8/Eae	1	<1

[a] Only the F17 probe-positive strains confirmed by family PCR are presented.
[b] One strain was negative with the Afa probe, but positive with AfaE-8 probe.

between AfaE-8 and F17 probes (47 strains; 8.7%), in association with P or S probes in 10 strains, and the second most frequent combination was between P and S probes (13 strains; 2.4%), in association with F17 probe in two strains. The other combinations accounted for less than 1% each (Table 3).

Thus, 468 positive *E. coli* (86.7%) were positive with only one probe (Table 3): P (351 strains; 65%), S (52 strains; 9.6%), AfaE-8 (24 strains; 4.4%), F17 (22 strains; 4.1%) or Eae (19 strains; 3.5%).

3.4. Plasmid hybridisation

Plasmid DNA was extracted from all 75 F17- and all 76 Afa-8-positive *E. coli* and hybridised with the F17 and AfaE-8 probes. No positive results were obtained except for the plasmids extracted from the positive controls.

4. Discussion

Although several *E. coli* strains are responsible for systemic infections in poultry, their virulence factors, in particular their adhesins and putative colonisation factors are unknown for most. According to the results obtained by DNA–DNA colony hybridisation on 1601 *E. coli* isolated from internal organs and intestines of chickens, turkeys and ducks, Afa-VIII, F17, S (Sfa/F1C) and Intimin (Eae) adhesins may represent colonisation factors of some APEC, besides the well described P (Pap/Prs) fimbrial adhesins. All groups of probe-positive *E. coli* were represented almost equally in chickens, turkeys and, to a lesser extent, ducks (Table 1). The presence and prevalence of *pap*/*prs*-related DNA sequences in *E. coli* from chickens and turkeys is no surprise according to previous results (Dho-Moulin and Fairbrother, 1999). On the other hand, *Sfa*/*foc*-, *f17*-, *afa*-, and *eae*-related DNA sequences have previously been described only in a few Algerian and Irish chicken *E. coli* (Gérardin et al., 2000; Mellata et al., 2001).

As expected, the majority of P-, S-, F17- and Afa-positive strains (>85%) originated from extraintestinal organs (Table 1), but the positive strains of intestinal origin emphasise once more the intestinal carriage of avian (putative) pathogenic *E. coli* (Dho-Moulin and Fairbrother, 1999). Subtyping identified the *f17*- and *afa*-related DNA sequences present in avian *E. coli* as variants present in *E. coli* from mammals. The *f17*Ac and *f17*GII variants are the most frequent *f17* gene variants and the *afa*-8 is the only *afa* variant present in avian *E. coli*, as previously observed for *E. coli* from mammals (Bertin et al., 1996b; Le Bouguénec and Bertin, 1999; Gérardin et al., 2000; Mainil et al., 2000). In contrast to mammalian *E. coli* in which the *f17*c and *afa*-8 gene clusters can be plasmid-located (Mainil et al., 1997, 2000; Mainil, 1999; Gérardin et al., 2000), the *f17*- and *afa*-8-related DNA sequences are not plasmid-located in the poultry *E. coli*. No in vitro phenotypic assay was performed in this study, but the colony hybridisation and the subtyping results provide strong evidence for the presence of complete *f17*- and *afa*-8 gene clusters and thus for production of these adhesins. Indeed, several genes of the respective clusters were detected: *f17*A, *f17*G (at least in 60 *E. coli*) and part of *f17*D on the one hand; *afa*B, *afa*D-8 and *afa*E-8 on the other hand (Le Bouguénec and Bertin, 1999).

Although the associations between the *f17*A and *f17*G gene variants (*f17*Aa and *f17*GI, *f17*Ac and *f17*GII) are identical to those observed previously in mammalian *E. coli* (Bertin et al., 1996b), the detection and subtyping of *f17*-related sequences in variant *E. coli* calls for further comments. The 46 strains with weak hybridisation results and negative family PCR results may harbour new variant(s) of the *f17* gene clusters; similar results (Mainil et al., 1997) led to the description of the *afa*-7 and *afa*-8 gene clusters in bovine *E. coli* (Lalioui et al., 1999). The strains with untypeable *f17*A and/or *f17*G genes (Table 2) may harbour new variants of these genes, as previous results already suggested (Bertin et al., 1996b). Alternatively the 15 strains with untypeable *f17*G genes may harbour incomplete *f17* gene clusters, as already observed for *pap*/*prs* gene clusters in APEC (Dho-Moulin and Fairbrother, 1999). The few strains positive for two *f17*A variants may actually harbour two *f17* gene clusters, as already observed for mammalian *E. coli* (Bertin et al., 1996b).

A few avian *E. coli* only were positive with the Eae probe derived from the *eae* gene. This one codes for the Intimin adhesin, which is part of the pathogenicity island called Locus of Enterocyte Effacement (LEE) of EPEC and EHEC and involved in the production of attaching/effacing (A/E) lesion (Nataro and Kaper, 1998). Several of them were recovered from intestinal sites, but the majority (65%) were unexpectedly isolated from internal organs (Table 1). None were positive for the production of verotoxin (Blanco and Oswald, unpublished results) and all correspond to EPEC, rather than to EHEC, although they were negative with the Bfp probe derived from a gene cluster coding for an initial colonisation factor of human EPEC (Nataro and Kaper, 1998). It may thus be speculated that avian EPEC produce their own specific primary colonisation factor(s). PCR subtyping of the *eae* gene detected the β- and the γ-variants, results similar to those obtained on a majority of EPEC and EHEC isolated from mammals (excluding man) (China et al., 1999a; Oswald et al., 2000; Goffaux et al., 2000). Presence of a complete LEE in these avian *E. coli* will be investigated using PCR assays for other LEE-located genes (China et al., 1999a).

Several associations among different gene clusters were observed between the *pap*/*prs*- and the *sfa*/*foc*-, *f17*-, *afa*-8-, and/or *eae*-related DNA sequences. The most frequent association was actually between the *f17*- and *afa*-8-related gene clusters (Table 3), as in NTEC2 strains from cattle (Mainil et al., 1997, 1999), although the poultry *E. coli* were non-NTEC (Blanco and Oswald, unpublished results). The presence of gene clusters coding for different adhesins is quite common in mammalian *E. coli* (Smyth et al., 1994), although the exact reason is not known. The expression of several adhesins by APEC would enhance their capability to colonise mucosae, perhaps even different mucosae, such as the intestine, which constitutes the reservoir, and the respiratory tract, which represents the entry site before invasion. This speculation needs confirmation with in vivo experiments.

F17, S fimbrial, Afa-VIII, and Intimin afimbrial adhesins may thus represent new virulence factors of APEC. Future experiments must be conducted to answer the following questions: (i) are these adhesins expressed in vitro and in vivo; (ii) are these *E. coli* specific pathogens for chickens, turkeys or ducks, after inoculation into the respiratory or intestinal tracts; and (iii) are these adhesins specific poultry colonisation factors for the respiratory or intestinal tracts.

In vitro (with genetic and phenotypic assays, immunological assays and cell cultures) and in vivo (with ligated intestinal loop assays and tracheal challenge in young chickens, turkeys or ducks) studies with wild-type and allelic mutants will bring answers to these three questions.

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