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High prevalence iron receptor genes of avian pathogenic *Escherichia coli*

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Abstract

Avian Pathogenic *Escherichia coli* (APEC) are known to cause significant losses in the poultry industry worldwide. Although prophylactic measures based on vaccination are advisable, until now no full heterologous protection against colibacillosis has been achieved. Since iron is an essential nutrient to these bacteria, the aim of this study was to investigate the prevalence of 12 outer membrane iron receptor genes in 239 pathogenic strains isolated from clinical cases of colibacillosis in chickens. Five multiplex PCRs were developed as a tool for efficient screening. Among the 239 avian *E. coli* isolates, 100% were positive for *fhuE* and *fepA*, 96.2% for *fiu*, 92.9% for *cir*, 92.5% for *iroN*, 87.4% for *iutA*, 63.2% for *fecA*, 53.1% for *fyuA*, 46.9% for *fhuA*, 45.6% for *ireA*, 41.8% for *chuA* and 4.6% for *iha*.

Introduction

Avian pathogenic *Escherichia coli* (APEC) cause disease in poultry worldwide leading to major economic losses (Gross, 1994; Dho-Moulin & Fairbrother, 1999).

One of the virulence factors is the iron-sequestration system. *E. coli* have been found to contain at least 11 outer membrane ferri-siderophore receptors (Cir, FecA, FepA, FhuA, FhuE, FiuA, FyuA, Iha, IreA, Iron and IutA) and one receptor for haem (ChuA) (Wagegg & Braun, 1981; Hantke, 1981, 1983; Fecker & Braun, 1983; de Lorenzo *et al.*, 1986; Pierce & Earhart, 1986; Torres & Payne, 1997; Schubert *et al.*, 1998; Russo *et al.*, 2001; Hantke *et al.*, 2003; Léveillé *et al.*, 2006).

To identify the most prevalent OM receptors involved in iron uptake, 5 multiplex PCR's were developed to investigate 239 APEC strains recovered from colibacillosis-affected chickens.

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Materials and Methods

Bacterial strains and media. The 239 strains collected from different farms and outbreaks were isolated from blood or organs from chickens showing distinct clinical symptoms of APEC infection and were kindly provided by DGZ (Drongen, Belgium, 175 strains) and Intervet International (Boxmeer, The Netherlands, 64 strains).

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O serogrouping of the Belgian strains was performed as described by Vandemaele *et al.* (2003). The Dutch strains were O serogrouped by agglutination with reference antisera at the National Institute of Public Health (RIVM), Bilthoven. For 155 of the strains, the chicken type of origin is known: 76 strains from layers (serogroups: 2 x O1, 11 x O2 and 25 x O78), 64 from broilers (serogroups: 3 x O1, 6 x O2 and 25 x O78) and 15 from breeders (serogroups: 4 x O1, 1 x O2 and 3 x O78). O1, O2 and O78 serogroup distribution in the remaining 84 strains was 1, 6 and 10 respectively. Strains were grown in standard Luria-Bertani (LB) medium at 37°C at 230 rpm and stored at -80°C in LB medium containing 15% glycerol.

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Multiplex PCR. Five multiplex PCRs were designed to detect the iron receptor genes (Table 1) using APEC1 (Vandemaele *et al.*, 2006) and APEC25, a strain of the O109 serotype isolated from the peritoneum of a deceased broiler, as reference strains.

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Two µl of bacterial LB overnight culture were added to a 18 µl PCR master mix composed of Supertaq buffer (SphaeroQ, Leiden, The Netherlands), 0.75U Supertaq DNA polymerase (SphaeroQ), 0.2 mM dNTP (Roche Diagnostics, Mannheim, Germany), 5% DMSO (Invitrogen, Paisley, Scotland), 1 µM of each primer (Table 1; Invitrogen)

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and DNase free water. The PCR conditions were initiated with 93°C for 10 min and followed by 35 cycles each consisting of denaturation at 94°C for 1 min, the annealing temperature given in Table 1 for 1 min and extension at 72°C for 1 min. The PCR was terminated by a final elongation for 10 min at 72°C. All PCR products were visualized on a 2% agarose gel containing SYBRsafe by transillumination. DNase free water was used as negative control.

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Statistical analysis. All statistical analyses were performed using the SAS software vs. 8.2 (SAS institute Cary, NC, USA). The Univariate procedure and the Kruskal-Wallis analysis were applied and the significance level was set to $\alpha = 0.05$.

Results and Discussion

The multiplex PCRs gave clear PCR products (Figure 1): 92.5% of the strains were positive for *iroN*, 87.1% for *iutA*, 41.8% for *chuA*, 45.6% for *ireA* and 4.6% for *iha*.

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Similar prevalence rates were reported by Rodriguez-Siek *et al.* (2005) (89.7% *iroN*, 82.2% *iutA*, 44.4% *ireA* and 3.4% *iha*), by Ewers *et al.* (2007) (83.7% *iroN*, 41.3% *ireA*, 3.1% *iha* and 50.3% *chuA*) and by Vandekerchove *et al.* (2005) (90% prevalence for

jutA). *FyuA* could be found in 53.1% of our APEC strains while other research groups found this gene in 58.8% (Rodriguez-Siek *et al.*, 2005), 66.4% (Ewers *et al.*, 2007), 71.3% (Ewers *et al.*, 2004) or 90% (Vandekerchove *et al.*, 2005) of the APEC strains

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from chickens. The suggestion that *fyuA* is part of an unstable pathogenicity island (Rakin *et al.*, 1995) might explain these differences. The prevalence of *fepA* (100%), *fhuE*

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(100%), *fiu* (96.2%), *cir* (92.9%), *fecA* (63.2%) and *fhuA* (46.9%) in APEC strains has not been published before.

As broilers are more sensitive to APEC compared to other *E. coli* (Gross 1994) and O1, O2 and O78 are considered to be the predominant serogroups associated with infection (Dho-Moulin & Fairbrother, 1999) (although this is contradicted by more recent studies (Rodriguez-Siek *et al.*, 2005)), the correlation between type of bird or serogroup on one hand and gene prevalence on the other hand was investigated. The prevalence of *chuA*, *fhuA*, *fyuA* and *ireA* was significantly higher in strains containing the O1 or O2 antigens than in strains containing the O78 antigen (Table 2). *Iha* was significantly more present in strains of the O1 serogroup than in strains of the O2 or the O78 serogroups. In contrast, *fecA* was more prevalent in strains belonging to the O78 serogroup followed by strains from the O2 and eventually by strains from the O1 serogroup. The *fecA* and *iutA* genes were significantly more present in strains from the O1+O2+O78 group (containing either O1, O2 or O78 antigens) than in strains not belonging to these serogroups. *FhuA* was significantly less prevalent among the O1+O2+O78 group.

Both *fyuA* and *chuA* were more frequently detected in strains isolated from layers (56.6% and 47.4%) and breeders (66.7% and 73.3%) than in those from broilers (32.8% and 23.4%) while *fiu* was significantly more prevalent in strains from broilers (98.4%) than in those from breeders (86.7%). For *fyuA* and *chuA* these differences could be partially related to their correlation with the O1, O2 and O78 serogroups.

In conclusion, *fhuE*, *fepA*, *fiu*, *cir*, *iroN* and *iutA* are the most abundant in APEC strains. Interfering with these OM receptors may prove valuable for the protection of chickens against colibacillosis.

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Acknowledgements

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Sincere thanks to Karolien Loots for help with statistics and discussion.

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induces strong adhesion-inhibiting antibody responses but not protection against avian pathogenic *Escherichia coli*. *Avian Pathology*, 35, 238-349.

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Figure legends

Figure 1: *Detection of the iron receptor genes by multiplex PCR. Each picture shows the negative control (DNase free water) in the first lane, the molecular marker Smartladder SF (Eurogentec) with 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp and 1000 bp fragments in the middle lane and the positive control (APEC25 for iha and fiu and APEC1 for all other genes) in the last lane.*

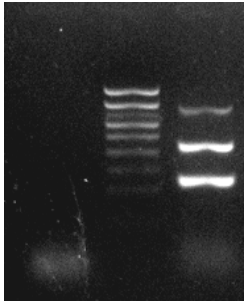
Table 1 The primer sequences, sizes of amplified fragments, and PCR conditions used for the detection of the iron receptor genes

Multiplex	Gene	Primers	Length of PCR product (bp)	Annealing temperature
1	<i>iroN</i>	IroN_F: 5' ctctggtggtggaagccacc 3' IroN_R: 5' tgcggtacagcggttcgctc 3'	814	64°C
	<i>iutA</i>	IutA_F: 5' ctgcagtactccgatcggctg 3' IutA_R: 5' tggttggaggtaaagcgctcatg 3'	470	
2	<i>fhuA</i>	FhuA_F: 5' agacactatcacccgttaccgctg 3' FhuA_R: 5' gccgcgaatgatcaggtggtc 3'	265	56.5°C
	<i>fyuA</i>	FyuA_F: 5' acggctttatcctctggccttg 3' FyuA_R: 5' tgaaaacccagtcacggtgg 3'	878	
3	<i>fepA</i>	FepA_F: 5' agctgactgacagcaccatcg 3' FepA_R: 5' cgggatgatcgaaaacggtcg 3'	554	68°C
	<i>chuA</i>	ChuA_F: 5' gttacggcaacggggaatgc 3' ChuA_R: 5' agcagggtaatcggcagatcg 3'	1061	
4	<i>fecA</i>	FecA_F: 5' aggttaatatcgaccgggatcg 3' FecA_R: 5' atggcatccatgttgccgagc 3'	565	56°C
	<i>ireA</i>	IreA_F: 5' tgttactgcctccgggttcac 3' IreA_R: 5' ccggttttcattcccaccagtg 3'	158	
5	<i>cir</i>	Cir_F: 5' tgtcactgcacttccgtggaac 3' Cir_R: 5' tgcagcaagtcggaatcacg 3'	704	55°C
	<i>fhuE</i>	FhuE_F: 5' gtcggtcactattgttagccagc 3' FhuE_R: 5' aattcacgactggtcgctgtg 3'	330	
5	<i>fiu</i>	Fiu_F: 5' ccggtcttgtattggcatcacg 3' Fiu_R: 5' acgattcgctgtaccaaggcca 3'	689	55°C
	<i>iha</i>	Iha_F: 5' ttactggcatgccgaggcagt 3' Iha_R: 5' cggatgctgattccagcccac 3'	410	

Table 2: Prevalence of iron receptor genes in relation to O-serogroup

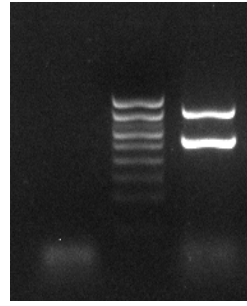
iron receptor gene	Iron receptor genes (%)			Iron receptor genes (%)	
	O1 (n=10)	O2 (n=24)	O78 (n=63)	O1+O2+O78 (n=97)	Other (n=142)
<i>chuA</i>	100 ^(a)	87.5 ^(a)	7.9 ^(b)	37.1	45.0
<i>fhuA</i>	90.0 ^(a)	66.7 ^(a)	11.1 ^(b)	33.0*	56.3*
<i>ireA</i>	90.0 ^(a)	75 ^(a)	36.5 ^(b)	51.4	41.5
<i>fyuA</i>	90.0 ^(a)	87.5 ^(a)	44.4 ^(b)	59.8	48.6
<i>iha</i>	40.0 ^(a)	4.00 ^(b)	0.00 ^(b)	5.20	4.00
<i>fecA</i>	20.0 ^(a)	75 ^(b)	93.7 ^(c)	81.4*	50.7*
<i>cir</i>	90.0	100	90.5	92.8	93.0
<i>fiu</i>	90.0	95.8	96.8	95.9	96.5
<i>iroN</i>	90.0	100	95.2	95.9	90.1
<i>iutA</i>	100	100	96.8	97.9*	80.3*
<i>fepA</i>	100	100	100	100	100
<i>fhuE</i>	100	100	100	100	100

Significant differences ($P \leq 0.05$) in prevalence for each individual iron receptor gene between strains of the O1, O2 and O78 serogroups are indicated with a different letter. Significant differences ($P \leq 0.05$) in prevalence for each individual iron receptor gene between the total of strains containing one of these O1, O2 or O78 LPS antigens and strains belonging to any other serogroup are indicated with an asterix.



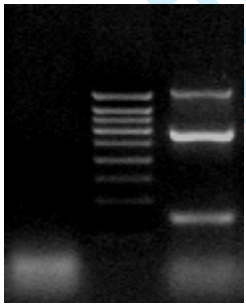
← *iroN* (814 bp)
← *iutA* (470 bp)
← *fhuA* (265 bp)

Multiplex 1



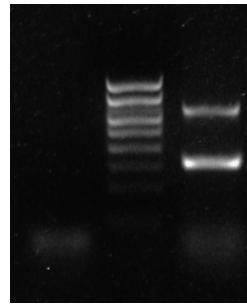
← *fyuA* (878 bp)
← *fepA* (554 bp)

Multiplex 2



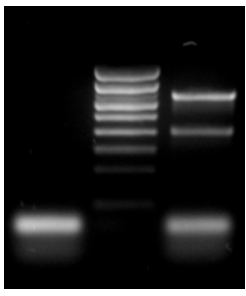
← *chuA* (1061 bp)
← *fecA* (571 bp)
← *ireA* (158 bp)

Multiplex 3



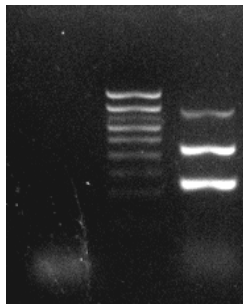
← *cir* (704 bp)
← *fhuE* (330 bp)

Multiplex 4



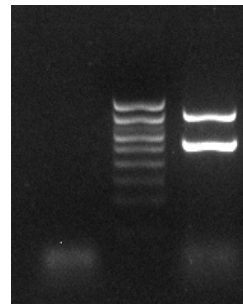
← *flu* (689 bp)
← *iha* (410 bp)

Multiplex 5



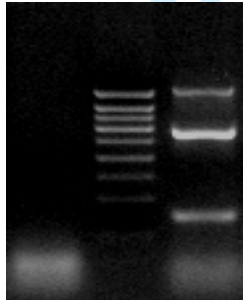
← *iroN* (814 bp)
← *iutA* (470 bp)
← *fhuA* (265 bp)

Multiplex 1



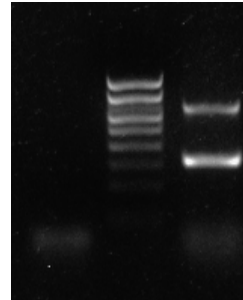
← *fyuA* (878 bp)
← *fepA* (554 bp)

Multiplex 2



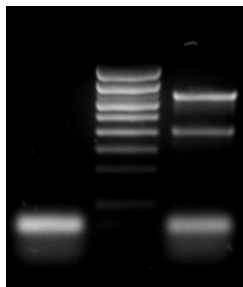
← *chuA* (1061 bp)
← *fecA* (571 bp)
← *ireA* (158 bp)

Multiplex 3



← *cir* (704 bp)
← *fhuE* (330 bp)

Multiplex 4



← *fiu* (689 bp)
← *iha* (410 bp)

Multiplex 5