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Emerging avian pathogenic *Escherichia coli* strains belonging to clonal groups O111:H4-D-ST2085 and O111:H4-D-ST117 with high virulence-gene content and zoonotic potential

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ABSTRACT

The present study characterizes, for the first time, two emerging avian pathogenic *Escherichia coli* (APEC) clonal groups of serogroup O111: O111:H4-D-ST117 and O111:H4-D-ST2085. The clonal group O111:H4-D-ST117 was already present in APEC strains isolated between 1991 and 2000, and was still present in strains isolated between 2004 and 2009, showing long time evolution according to the virulence-gene differences and macrorestriction profiles. Among ST117 strains, two virulence profiles could be distinguished: *papG II*-positive *tsh*-negative strains which satisfied criteria for extra-intestinal pathogenic *E. coli* (ExPEC), and *papG II*-negative *tsh*-positive strains without ExPEC status. Interestingly, we have detected a human septicemic O111:H4-D-ST117 ExPEC strain isolated from a hemocultive in 2000 whose macrorestriction profile showed >85% similarity with four APEC strains of the study. The clonal group O111:H4-D-ST2085 was exclusively detected in 17 APEC strains isolated in 2008 and 2009, and showed short time evolution based on its homogeneity since all were nalidixic acid-resistant, all had ExPEC status, and most carried *papG II* and *tsh* genes. From the clinical point of view, O111:H4-D-ST2085 seems a successful clonal group that could be the result of the epidemiological evolution of O111:H4-D-ST117. Due to the increasing prevalence of both clonal groups among clinical APEC isolates, their high virulence-gene content, and zoonotic potential, we suggest them as possible candidates for the development of a future vaccine against avian colibacillosis.

1. Introduction

Avian colibacillosis is an infectious disease of birds caused by *Escherichia coli*. It is considered one of the leading causes of morbidity and mortality associated with severe economic losses in the poultry industry for their involvement in different disease conditions, either as primary or secondary pathogen (Barnes and Gross, 1997).

E. coli is considered a member of the normal gut microflora of birds, but some strains designated as avian pathogenic *E. coli* (APEC) are pathogenic due to the acquisition of virulence factors that confers the ability to colonize the internal organs and produce avian colibacillosis. Multiple serogroups are associated with this disease, especially O1, O2 and O78, among many others (Blanco et al., 1997, 1998; Dho and Fairbrother, 1999).

APEC strains are included in the group of extraintestinal pathogenic *E. coli* (ExPEC) together with human uropathogenic *E. coli* (UPEC), septicemic *E. coli*, and newborn meningitis-causing *E. coli* (NMEC). Although ExPEC exhibit

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considerable genetic diversity with a wide range of virulence factors, numerous studies have shown that APEC share common characteristics with human pathogenic strains, including the K1 capsular antigen gene and the *ibeA* gene, among others (Moulin-Schouleur et al., 2006, 2007). Even some studies have shown that certain APEC strains belong to the same clonal groups than human ExPEC strains (Mora et al., 2009; Tivendale et al., 2010), emphasizing their potential as zoonotic agents.

We have detected an increasing prevalence of serogroup O111 among APEC strains recently isolated in Spain (from 0% of 536 APEC isolated between 1991 and 2000 to 7.9% of 1101 APEC isolated between 2004 and 2009). Therefore, we decided to characterize recent O111 APEC strains and compare them with old isolates from other European countries to know if a new clonal group is emerging, and if so, which its pathogenic potential is.

2. Materials and methods

2.1. Bacterial strains

According to our data, while serogroup O111 accounted globally for 0.7% of 1601 avian colibacillosis isolates collected in Europe (536 strains from Spain, 974 from France and 91 from Belgium) between 1991 and 2000 (Stordeur et al., 2002), this serogroup accounted for 7.9% of 1101 APEC isolated in Spain between 2004 and 2009. Furthermore, none of the 536 Spanish strains from the first collection belonged to serogroup O111 ($P = 0.000$). Therefore, O111 was identified as an emerging serogroup among avian colibacillosis in Spain, and this was the first criterion for selection of the strains of the present study.

Initially, 11 O111 strains from the european collection (1991–2000) and 47 O111 strains from the Spanish collection (2004–2009) were analyzed for their phylogroup (one isolate per animal and farm). Finally, only O111 strains belonging to phylogroup D were further characterized.

Finally, the present study included 48 O111-D strains (47 avian and one human). The 47 APEC strains had been obtained from clinical cases of colibacillosis from two collections isolated during 1991–2000 (five strains) and 2004–2009 (42 strains). The five APEC strains of first collection were isolated in Belgium (two strains isolated from chicken: APEC 0037, APEC 0688) and France (two strains isolated from chicken: APEC 0081, APEC 0115; and one from turkey: APEC 0110). The 42 APEC strains of second collection came from different geographic areas of Spain, and all of them had been isolated from chicken. Additionally, one human septicemic O111-D strain isolated in 2000 and obtained from a Spanish collection of 3050 hemocultive strains was included in the study.

2.2. O and H typing

Determination of O and H antigens was carried out using the method previously described by Guinée et al. (1981) with all available O (O1–O181) and H (H1–H56) antisera. Isolates that did not react with H antisera were classified as non-typeable (HNT), and those non-motile were denoted as HNM.

Those strains HNM or HNT were tested by PCR to detect the presence of the flagellar H4 gene using two oligonucleotide primers designed by us (*fliC*-H4-F 5′-GCAGCGTATTCGTGAACTGA-3′ and *fliC*-H4-R 5′-TGAAACGACACCACTTATTGC-3′) to amplify a fragment of 713 bp.

2.3. Phylogenetic analysis and multilocus sequence typing (MLST)

Strains were assigned to one of the four main phylogenetic groups of *E. coli* (A, B1, B2 and D) by using the multiplex PCR-based method of Clermont et al. (2000). Only those strains belonging to phylogroup D were further characterized.

Among the 48 O111-D *E. coli* strains, and based on the similarities of the macrorestriction profiles, a representative selection of 13 strains was analyzed by MLST, so that the Sequence Types (STs) of strains from all clusters were established. MLST was achieved as previously described by gene amplification and sequencing of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) according to the protocol and primers specified at the *E. coli* MLST web site (http://mlst.ucc.i.e./mlst/dbs/Ecoli). The allelic profile of the seven gene sequences, the STs, as well as the Sequence complexes (defined as STs that are linked by distances of one or two allelic differences) were obtained via the electronic database at the *E. coli* MLST web site (Martinez-Medina et al., 2009).

2.4. Antimicrobial susceptibility and extended-spectrum beta-lactamase (ESBL) typing

Susceptibility to antibiotics was analyzed by broth microdilution. Minimal inhibitory concentrations were determined using a MicroScan WalkAway automated system (Siemens, Madrid, Spain) according to the manufacturer's instructions. Intermediate susceptibility was not considered as resistant. Resistance was interpreted based on the recommended breakpoints of the CLSI (CLSI, 2010). Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of cefotaxime, ceftazidime, aztreonam or cefepime. To determine the genotype of the ESBLs, PCR was performed using the TEM, SHV, CTX-M-1 and CTX-M-9 group-specific primers, as reported previously (Leflon-Guibout et al., 2004; Blanco et al., 2009).

2.5. Virulence genotyping

The presence of virulence genes was analyzed as documented previously (Mora et al., 2009; Blanco et al., 2011) using primers specific for genes and operons that encode virulence factors characteristic of ExPEC. These genes included those for adhesins (*fimH*; $fimAv_{MT78}$; *papEF* with positive results tested for *papG* I, *papG* II, *papG* III and *papG* IV alleles; *sfa/focDE* with positive results tested for *sfaS* and *focG*; *afa/draBC*; *bmaE*; *gafD*); toxins (*cnf1*; *cdtB*; *sat*; *hlyA*); siderophores (*iucD*; *iroN*); protectins/invasion genes (*kpsM* II establishing neuC-K1, -K2 and -K5 variants; *kpsM* III; *cvaC*; *iss*; *traT*; *ibeA*); and miscellaneous genes (*malX*; *usp*; *tsh*).