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Clonality and virulence traits of *Escherichia coli* associated with hemorrhagic septicaemia in turkeys.

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Abstract

Fifty-five clinical isolates of avian pathogenic *Escherichia coli* (APEC) from seven outbreaks of acute hemorrhagic septicaemia in turkeys were characterised by serotyping, plasmid profiling including restriction analysis with *Hind*III, ribotyping with *Eco*RI and *Hind*III, multilocus sequence typing (MLST) and virulence profiling. A clonal relationship was demonstrated for each outbreak according to serotype, plasmid profiling, ribotyping, and MLST. In addition, isolates demonstrated highly similar virulence profiles, as all isolates were positive for F11 pili and possessed genes encoding aerobactin (*iucD*), increased serum survival (*iss*), temperature-sensitive hemagglutinin (*tsh*) and colicin V plasmid operon genes (*cva/cvi*). However, only 20% of the isolates produced colicin V and 42% exhibited serum resistance. All strains with O group O111 and a single O18ac strain (demonstrating non-clonal DNA profiles) were positive for enteroaggregative heat stable toxin (EAST1), while isolates of a single outbreak all possessed the enteroaggregative toxin gene (*astA*). All isolates were negative for genes encoding verocytotoxins (*vtx/stx*), iron-repressible protein (*irp2*), P-fimbria (*papC*), invasion plasmid antigen (*ipaH*), attaching and effacing gene (*eae*), enterohaemolysin (*ehxA*), and enterotoxins LT, STIa (ST_p) and STIb (ST_h). In conclusion, highly similar virulence profile were demonstrated for isolates of *E. coli* associated with a single well-defined lesion type of colibacillosis in turkeys; acute hemorrhagic septicaemia. Isolates obtained, however, demonstrated a different phylogenetic background, underlining the importance of using well-defined strain collections for characterization of APEC pathotypes.

Introduction

Infections of domestic birds caused by *Escherichia coli* result in a variety of pathological manifestations, depending on the organs infected. Collectively, these extra-intestinal infections are referred to as colibacillosis, a disease responsible for major economic losses to the poultry industry in most parts of the world (Dziva & Stevens, 2008; Barnes *et al.*, 2008). Colibacillosis in poultry includes a variety of localized or systemic infections in which *E. coli* may be involved as the primary or secondary infectious agent (Barnes *et al.*, 2008; Rodriguez-Siek *et al.*, 2005). Avian pathogenic *E. coli* (APEC) isolates are commonly reported to belong to certain O groups, particularly O1, O2, O8, O15, O18, O35, O78, O88, O109 and O115 (da Silveira *et al.*, 2002). In domestic poultry, colibacillosis is frequently associated with *E. coli* serotypes O78:K80, O1:K1, O2:K1 and O group O35 (Ewers *et al.*, 2003).

Previously, ribotyping and serotyping were commonly used for characterization of *E. coli* (da Silveira *et al.*, 2003). Recent studies, however, have demonstrated variations in major outer membrane proteins and metabolic enzymes demonstrating that prevalent O groups are genetically diverse and of different clonal origin (Ron, 2006). Multilocus sequence typing (MLST) has subsequently emerged as an excellent tool for evaluation of population clonality. In addition, unambiguous comparison of sequence types (STs) is possible through the internet-based database (www.mlst.net) (Perez-Losada *et al.*, 2006).

Several potential virulence factors have been associated with avian pathogenic *E. coli* (APEC) including type 1 fimbriae (La Ragione & Woodward, 2002); curli fimbriae (La Ragione & Woodward, 2002); P fimbriae (F11) (Mellata *et al.*, 2003); AC/1 fimbriae and type-1-like fimbriae (Nakazato *et al.*, 2009); flagella (La Ragione *et al.*, 2000); capsular antigen (K1) (Mellata *et al.*, 2003); temperatur sensitive haemagglutinin (*tsh*) (Janssen *et al.*, 2001) and possession of high

molecular weight plasmids associated with colicin V genes and aerobactin iron scavenging systems.

Even though several studies have been undertaken, which show that the virulence factors described above are involved, either alone or in combination, *E. coli* is still commonly reported as an opportunistic pathogen which causes disease in cases of decreased host resistance. If so, major diversity is expected to be associated with outbreaks, but an increased significance, and a primary (with clonal outbreaks) rather than secondary role of APEC in layers has recently been reported (Vandekerchove *et al.*, 2004; Gregersen *et al.*, 2011). The discovery and characterization of virulence factors associated with pathogenic clones might lead to a better understanding of the pathogenesis of infectious diseases and provide tools for efficient therapy and prevention of these diseases. Little is, however, known/published concerning the diversity of *E. coli* populations within the flock during outbreaks of defined disease. Previous attempts to define the APEC pathotype have encountered major difficulties (Rodriguez-Siek *et al.*, 2005), but this might to some extent be explained by neglecting the importance of well-characterized strain collections. Many studies investigating the APEC population state “colibacillosis” as the only pathological description of the lesion type from which the isolates were originating (Johnson *et al.*, 2008b; Mellata *et al.*, 2003). Yet, this term is merely a common denominator for a variety of lesion types, in which a number of different combinations of virulence factors might be applied (Liebchen *et al.*, 2008).

The purpose of this study therefore was to characterize a number of clinical isolates from outbreaks of colibacillosis in turkeys demonstrating a well-defined lesion type; acute hemorrhagic septicemia. The isolates were obtained from seven epidemiologically independent flocks over a period of two years with the aim to determine if clinical isolates were diverse or clonal, and whether they were associated with specific virulence factors common to APEC strains.

Materials and Methods

Bacterial strains. Fifty-five *E. coli* strains were isolated in pure culture from turkeys originating from seven epidemiologically and geographically unrelated outbreaks in flocks in Denmark during a two years period. The number of strains originating from each outbreak is stated in detail in Table 1. All birds exhibited clinical signs of sepsis. All strains were isolated in pure culture and demonstrated abundant growth in samples from liver, spleen, lung or pericardium. All strains were stored as Luria-Bertani (LB) broth (LB broth base [Gibco BRL]) cultures with sterile glycerol (15% [vol./vol.]) at -80°C. Before characterisation, the strains were propagated in LB broth and plated on to LB agar (Maniatis *et al.*, 1982).

Serotyping. Serotyping was carried out at The International *Escherichia* and *Klebsiella* Centre (WHO) in Copenhagen using methods described by Oerskov and Oerskov (1984). Briefly, O and H antigens were determined by bacterial agglutination in 170 O antisera and 52 H antisera, respectively. The presence of K antigen was assayed by the ability of a bacterial extract to precipitate with Cetavlon after electrophoresis. When the test was positive, the serotype of the K antigen was determined by counter current immunoelectrophoresis in 74 known K antisera. The presence of K1 and K5 was determined by K1 and K5 specific phages. Non-capsulated and non-motile strains were designated as K- and H-, respectively.

Plasmid analysis. Isolates of *E. coli* were screened for the presence of plasmid DNA using the method of Kado & Liu (1981). Gels were stained in ethidium bromide (Sigma), 2µg/ml, illuminated with ultraviolet light at 254nm using an UV transilluminator (UVP; model TS-40) and

photographed with a Polaroid Land Camera (Polaroid; MP-4). Plasmid motilities were calculated relative to the reference plasmids in *Escherichia coli* strains V517 (Macrina *et al.*, 1978) and 39R861 (Threlfall *et al.*, 1986) and sizes estimated in accordance with the methods of Rochelle *et al.* (1986). Coiled covalently closed (ccc) and open circular (oc) DNA molecules were differentiated according to Hintermann *et al.* (1981). Plasmid DNA restriction enzyme analysis, digestion with restriction endonuclease *HindIII* (Boehringer-Mannheim) and separation of restriction fragments were performed as previously described (Olsen, 1990). Phage lambda DNA (Boehringer-Mannheim), cut with *HindIII*, served as the molecular weight standard.

Ribotyping. Isolation of DNA and digestion with *EcoRI* and *HindIII* (Boehringer-Mannheim) and subsequent electrophoretic separation of the DNA fragments using agarose gel electrophoresis was performed as previously described (Christensen *et al.*, 1993). DNA was transferred to nylon hybridisation membranes (Hybond-N, Amersham) by vacuum blotting as recommended by the supplier (Pharmacia, LKB) and was fixed to the membrane by incubation at 80°C for 1 to 3 hours. Ribosomal RNA (16S and 23S) from *E. coli* (Sigma) was purchased and labelled with digoxigenin by the use of reverse transcriptase (Boehringer-Mannheim) as previously reported (Christensen *et al.*, 1993). The probes were hybridised with the membrane-fixed DNA under the conditions described by Christensen *et al.* (1993).

Multilocus sequence typing (MLST). Between two and six isolates from each outbreak were subjected to MLST. MLST was carried out according to (Gregersen *et al.*, 2010). In brief, amplifications of seven housekeeping genes (*adh*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were each carried out in a total volume of 50 µl using 1 µl of purified template DNA, 0.5 µl of each primer (20 pmol), 0.5 µl of the dNTPs (25 mM solution), 5 µl of 10×PCR buffer (Fermentas, Burlington,

Canada), 5 units of DreamTaq polymerase (Fermentas) and Millipore quality water to 50 µl. The PCR reaction conditions included a 2 min denaturation at 95 °C, 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 58 °C, extension 2 min 72 °C, and a final additional 5 min at 72 °C.

PCR products were purified using 10 µl PCR product, 1 µl calf intestine alkaline phosphatase and 1 µl Exonuclease I (Fermentas, Burlington, Canada). The mixture was incubated for 15 min at 37 °C and subsequently 15 min at 85 °C.

Multilocus sequence analysis. Sequencing was performed at MacroGen Inc., Seoul, Korea, using the PCR primers. Sequences were assigned sequence types based upon the scheme described at the *E.coli* MLST database (<http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli>), which assigns allele designations to unique variants based on a 500-bp internal region of each gene. A phylogenetic tree was constructed based upon the concatenated sequences of the housekeeping genes by the neighbour-joining method using MEGA 4.0.2 software (Tamura *et al.*, 2007).

Virulence genes. Single colonies and a sweep from an area with confluent growth were examined by colony or sweep dot blot hybridisation with the following pools of DNA probes: Pool for detection of verocytotoxin-producing *E. coli* (VTEC) (*vtx1*, *vtx2*) and enteroinvasive *E. coli* (EIEC) (Willshaw *et al.*, 1985; Thomas *et al.*, 1991) and invasion plasmid antigen (*ipaH*) (Venkatesan *et al.*, 1989), pool for the detection of enterotoxigenic *E. coli* (ETEC) (LT, ST_h & ST_p) (Sommerfelt *et al.*, 1988), pool for the detection of other diarrhoeagenic *E. coli* DNA (Bilge *et al.*, 1989), Eagg (Baudry *et al.*, 1990) and EAST1 (Savarino *et al.*, 1993), and single probes for the detection of plasmid encoded O157-enterohaemolysin (*ehxA*) (Levine *et al.*, 1987), enteropathogenic *E. coli* (EPEC) and attaching and effacing *E. coli* (A/EEC) (*eae*) (Jerse *et al.*, 1990). Single colonies reacting positively with the DNA probe pools were further characterised by hybridisation against the single

probes from the reacting pool of DNA probes. The probes were labelled with Digoxigenin-d-UTP (Boehringer) and colony blots were hybridised under stringent conditions according to the manufacturer's instructions. Detection of genes for P-fimbria (*papC*), aerobactin (*iucD*), iron-repressible protein (*irp2*), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), enteroaggregative toxin (*astA*), increased serum survival protein (*iss*), and colicin V plasmid operon genes (*cva/cvi*) was done according to Ewers *et al.* (2005).

Colicin production. The ability of strains to produce colicin V was assessed using the method of Blanco *et al.* (1997). Strains were streaked on to the surface of tryptone soy agar (TSA) (Oxoid) plates and incubated overnight at 37°C. Growth on the surface of the plates was killed by treatment with chloroform vapour for 30 minutes following which residual chloroform was allowed to evaporate in the fume cabinet. Warm, liquid TSA containing suspensions of the indicator organisms *E. coli* K12-711 (ColV⁺) or *E. coli* K12-711 negative (ColV⁻) was flooded over the surface of the plates and allowed to solidify and incubated overnight at 37°C. Strains were considered to produce colicin V if a zone of clearing was observed in the overlay of the 711 negative strain but not in the overlay of 711 positive strain.

Serum resistance. Turkey blood was obtained from healthy turkeys via the jugular vein and allowed to clot at room temperature. Serum was decanted, pooled and filtered through a 0.45 µm membrane filter and stored at -20°C until use. Overnight broths of the *E. coli* isolates and two control strains were centrifuged, 4000 rpm, 15 min and the pellets resuspended in an equal volume of phosphate-buffered saline (PBS). Two assays were carried out to determine survival and growth in the serum. Survival in serum was monitored for each organism by inoculating 150 µl of serum in the wells of a 96 well microtitre plate with a straight wire from the bacterial suspensions. This gave

an initial inoculum of approx 10^4 CFU ml⁻¹. The inoculated serum was incubated at 37°C and after 1, 3 and 6 hours, 20 µl was plated onto LB agar and colony counts compared with the control strains.

Strains were tested for their ability to grow in serum by a modification of the method used by Moll *et al.* (1979). For each strain tested, 30 µl of a solution of 2.5% glucose containing 0.25% bromothymol blue was pipetted into two wells of a 96-microtitre plate and dried at 37°C overnight. To one of the wells 120 µl of turkey serum was added and to the other 120 µl of PBS. Both wells were inoculated using a straight wire as before and the same control strains used. The tray was wrapped in a polythene bag and incubated for 18 hours at 37°C. Bacterial growth was indicated by the development of a yellow colour with a bacterial pellet in the bottom of the well. No growth was indicated by a blue colour.

F11 pili. Rabbit antisera against F11 were raised according to previous methods (Van den Bosch *et al.*, 1993) and preserved with 0.1% NaN₃. For determination of F11 antigen, enzyme-linked immunosorbent assay (ELISA) was carried out as described by Van den Bosch *et al.* (1993). Polystyrene flat-bottomed microtitre plates (Greiner) were coated overnight at room temperature with 100 µl of live cell suspension of *E. coli* per well with an OD_{660nm} of approx. 0.15. The plates were then blocked for 20 min at 37°C with 200 µl of PBS per well with 5% bovine serum albumin, washed with tap water and dried in air. Plates were incubated after rabbit antiserum was added in dilutions and later on the plates were incubated with 100µl of similarly diluted goat anti-rabbit conjugate. Bound antibodies were determined colorimetrically by adding 100 µl of enzyme substrate solution, containing 15 parts distilled water, 1.5 parts of 0.14% urea peroxide in sodium acetate-citric acid buffer (pH 5.5), and 0.2 parts of 0.6% 3,3'-5, 5'- tetramethyl benzidine (TMB) in dimethyl sulfoxide per well. The reaction was carried out in the dark and stopped after 10 min by

adding 50 µl of 2 M H₂SO₄ per well, and the A₄₅₀ was measured in a micro-ELISA reader. The antibody titre was defined as the maximum antiserum dilution with an A₄₅₀ of at least 1.5 times the mean A₄₅₀ background. All titres of ≥1:100 were considered positive.

Results

Pathology and bacteriology. All affected birds displayed a similar type of septicaemic lesions in the form of generalised circulatory disturbances, discolouration of subserosal fat in addition to hepato-, spleno- and renomegaly (Fig. 1a). Accumulation of blood and oedema characterized lesions in the lungs. Finally, serohaemorrhagic exudation was observed on serosal surfaces (Fig 1a). By incision, generalised liver necrosis was readily observed by the dry appearance of the cut-surface at *post mortem*, and the spleen often showed areas of localized necrosis (Fig. 1b). No indication of concurrent disease including endo- and ecto parasites in any of the birds was evident. All birds demonstrated pure culture of *E. coli* from liver, spleen and pericardium. Conclusively, each bird was assigned the diagnosis of acute, hemorrhagic septicaemia caused by *E. coli*.

Phenotypic analysis. Serotype analysis of the clinical isolates demonstrated that the majority of strains belong to the most prevalent types associated with APECs, i.e. O groups O1, O2, O18ac and O78 (Table 1). However, one other O group was also found, O111, not normally seen when typing APEC. Only a single serotype among isolates from the individual outbreaks was demonstrated, with the exception of outbreak six, where two different serotypes were evident. In addition, the same clone was demonstrated from different organs of the same turkey in 22 birds from which two or more isolates were characterized. Colicinogenicity occurred in 41 (75%) isolates, with 20 (36%) producing colicin V, while 23 out of 55 isolates (42%) exhibited serum resistance to the bactericidal activity of turkey serum. All 19 strains of O18ac:K1:H7 in outbreak seven were serum resistant and

did not produce ColV. In contrast, the two strains of O18ac:K1:H7 in outbreak six had colicin V and other colicin production. The K1 capsular antigen was found in serotypes O2:K1:H7 and O18ac:K1:H7 .

Genotypic analysis. Plasmid profiling of the strains showed that all clinical isolates possessed large plasmids (>100 kb) (Table 1). *Hind*III digestion of plasmids was used to demonstrate clonality of isolates (Fig. 2). Genotypical analysis by ribotyping with *Hind*III and *Eco*RI also confirmed the clonal nature of the *E. coli* outbreaks demonstrating identical ribotypes within outbreaks (Figs. 3 and 4). There was also some correlation between band pattern and serotype, although this was not exclusive as one serotype showed two different ribotypes (O18ac with ribotypes I/C and III/E) and two different serotypes showed the same ribotypes (O78 and O1 with ribotypes IV/D).

By MLST, all strains characterized within each outbreak were assigned the same ST (Table 1), substantiating the clonal nature of the APEC population involved in the single outbreaks. Outbreaks 1, 5 and 7 representing different serotypes all belonged to ST95, while outbreaks 2 and 3 shared both serotype and sequencetype (ST117). Outbreaks 6 and 7 represented identical serotypes, but belonged to different STs (ST23 and ST95, respectively). Outbreak 4 represented both a serotype and a ST (ST93) different from the remaining outbreaks. The four STs identified showed major phylogenetic diversity, and no clonal complexes among any of STs were demonstrated (Fig.5). The most distantly related STs (ST117 and ST23) differed in 54 nucleotides in the concatenated nucleotide sequences including all genes used in MLST, while the closest related STs (ST93 and ST23) differed in 25 nucleotides.

Characterisation of APEC associated virulence determinants. All clinical isolates demonstrated F11 and were positive for genes encoding aerobactin (*iucD*), increased serum survival (*iss*),

temperature-sensitive hemagglutinin (*tsh*) and vacuolating autotransporter toxin (*vat*). All strains of serotype O111:K:H4 and a single isolates of O18ac:K1:H7 strain (demonstrating non-clonal DNA profiles) were positive for enteroaggregative heat stable toxin (EAST1). Only isolates of outbreak 2 possessed the enteroaggregative gene, *asaA*. All isolates were negative for *vtx* genes, invasion plasmid antigen (*ipaH*), attaching and effacing gene (*eae*), O157-enterohaemolysin (*ehxA*), and enterotoxins LT, STIa (ST_p) and STIb (ST_h), for *vtx* (or *stx*) genes, iron-repressible protein (*irp2*) and P-fimbriae (*papC*).

Discussion

Major diversity has been reported as to lesion types of colibacillosis, the pathogenesis of which has remained unclear (Dziva & Stevens, 2008). Several studies have addressed differences between isolates obtained from lesions and isolates from apparently normal animals or the environment (Mcpeake *et al.*, 2005; Ewers *et al.*, 2003; Delicato *et al.*, 2003). Unfortunately, most of these studies did not state in detail the lesion type of colibacillosis investigated, which might to some extent explain the diversity reported and lack of specific virulence factors associated with colibacillosis.

In the present study isolates from seven epidemiologically unrelated outbreaks of colibacillosis in turkeys demonstrating a unique lesion type were characterized by several genetic methods, all of which demonstrated clonality of the single outbreaks. The clonality of the outbreaks suggests a primary rather than secondary role of *E. coli* as reported previously (Gregersen *et al.*, 2011; Vandekerehove *et al.*, 2004). However, four different clonal lineages were demonstrated, indicating that common virulence factors are more likely to be carried on large plasmids rather than

chromosomally encoded (Mellata *et al.*, 2010; Johnson *et al.*, 2008a). The hypothesis is further underlined as all isolates each possessed at least three genes (*iss*, *iucD* and *cva/cvi*) all carried primarily on plasmids (Johnson *et al.*, 2008c; Ewers *et al.*, 2005).

The clones demonstrated phylogenetic lineages (e.g. ST95) and serotypes of APEC including O groups O1, O2, O18 and O7, all commonly associated with APEC. The only exception was the clone demonstrating serotype O111, which occurred in both outbreaks 2 and 3, not often associated with APEC. This serotype has been reported in humans and various animal species (Morabito *et al.*, 1999; White *et al.*, 2002). These studies demonstrated a number of different ribotype patterns of O111 with varying virulence traits. The clone O111:H4 reported in this study exhibited a distinct ribotype pattern, but similar virulence profile with the isolates of other serotypes. Two of the

APEC serotype O groups found, O2 and O18, also demonstrated possession of capsule K1. This has previously been indicated as an important virulence factor in avian septicaemia (Bree *et al.*, 1989), but was only found in two of the six pathogenic serotypes obtained from acute, hemorrhagic septicaemia.

Despite the phylogentic and serotypic diversity between the outbreaks, virulence profiles based on the presence and absence of twelve virulence determinates were remarkable similar. Only minor differences in the virulence profiles among the fifty-five isolates was observed; a single gene, *astA*, was only demonstrated among isolates from outbreak two, and the EAST1 was only demonstrated among two serotypes.

Our results demonstrated a lack of geno- and phenotypic congruence. All isolates carried the colicin V operon genes, however, colicinogenicity only occurred in 41 (75%) of the isolates, with only 20 (36%) producing colicin V. This is comparable with a previous study of colibacillosis in turkeys (Emery *et al.*, 1992). It has previously been suggested that colicin V production, mediated

by ColV plasmids was mainly responsible for the pathogenicity of avian *E. coli* strains (Skyberg *et al.*, 2008). Our findings showed a number of colicin V producing strains similar to that found by Smith (1974) and Vidotto *et al.* (1991).

Plasmids carrying colicin V gene also carry other genes, which are responsible for the pathogenic traits observed in these strains (Johnson *et al.*, 2006). These genes would enable the strains to overcome the iron-limiting conditions existing in the host-body fluids by producing an iron-acquisition system, which is inducible by the low levels of body-circulating iron (Mellata *et al.*, 2009). It should be noted that strains in this study were not tested for their ability to produce aerobactin for iron acquisition, however, as all strains possessed the gene for aerobactin (*iucD*), it is likely that a high proportion of these strains are capable of aerobactin production, as shown previously in a study where 74% of turkey colisepticemia strains were positive for aerobactin (Emery *et al.*, 1992).

Another characteristic that contributes to pathogenicity of septicaemic strains is serum resistance. This can be due to the presence of conjugative plasmids, which express the outer membrane protein TraT, responsible for the surface exclusion phenomenon and enabling the bacteria possessing it to be resistant to the bactericidal activity of serum (Moll *et al.*, 1979). Again, our results demonstrated a lack of geno- and phenotypic congruence, as all isolates possessed the gene for increased serum survival protein (*iss*), but only 23 strains (42%) out of 55 isolates exhibited resistance to the bactericidal activity of turkey serum, with the majority of the strains derived from outbreak seven, serotype O18ac:K1:H7. Although Vidotto *et al.* (1991) correlated pathogenicity of avian colibacillosis with serum resistance; we were unable to confirm these findings, as serum resistance was variable in the clinical isolates, even within the same sero- and ribotype.

In the pathogenesis of avian colibacillosis, a correlation between virulence and adherence to tracheal or pharyngeal epithelial cells has been reported (Kariyawasam & Nolan, 2009). Fimbriae are responsible for mediating this adherence and have been reported in connection with type 1 fimbriae in particular (de Pace *et al.*, 2010). Wooley *et al.* (1992) found that 100% of the APEC strains produced type 1 fimbriae as compared with 57.5% of the commensal strains. Van den Bosch *et al.* (1993) has reported other fimbriae; F11 potentially involved in colibacillosis, and has demonstrated its occurrence in 78% of clinical isolates with an even higher association (96%) in common APEC serotypes like O1:K1, O2:K1, O78:K80 and O35. In this study, 100% of the clinical isolates demonstrated type F11 fimbriae. However, other studies have demonstrated a low prevalence of pathogenic *E. coli* to possess the F11 fimbriae (Bell *et al.*, 2002; Gregersen *et al.*, 2010) suggesting the F11 is not a prerequisite for the virulence potential of APEC.

In conclusion, this study demonstrated major similarities between virulence profiles of APEC obtained from epidemiological independent outbreaks of acute hemorrhagic septicaemia in turkeys in spite of different phylogenetic background and serotypes of the isolates. The similarity of the virulence profiles is higher than reported in other studies stating colibacillosis as the source of isolates characterized. A more detailed description of the lesion types from the strain collection isolated might add to a more precise definition and understanding of APEC pathotypes, which are likely to have different phylogenetic origin. Further investigations are, however, needed to confirm this hypothesis.

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Figure Legends

Figure 1. *Pathology of the lesion type acute, hemorrhagic septicemia at post mortem examination.*

(a) Swelling of parenchymatae organs demonstrating hepatomegaly with serohaemorrhagic exudation on serosal surfaces. (b) Coagulation necrosis of liver, necrotic foci of spleen and pale myocardium of the heart.

Figure 2. *Total plasmid DNA restriction analysis using HindIII demonstrating identical digestion profiles within each of seven outbreaks (1-7) of colibacillosis in turkeys.*

m – phage lambda marker

Figure 3. *Ribotyping (EcoRI) of Escherichia coli isolates from turkeys suffering from acute hemorrhagic septicemia. Lanes I-V represent each of six EcoRI ribotypes (and a single undergroup, Ia) demonstrated from seven outbreaks. The association between EcoRI ribotype and each outbreak is stated in Table 1. m – lambda marker.*

Figure 4. *Ribotyping (HindIII) of Escherichia coli isolates from turkeys suffering from acute hemorrhagic septicemia. Lanes A-E represent each of five HindIII ribotypes demonstrated from seven outbreaks. The association between HindIII ribotype and each outbreak is stated in Table 1. m – lambda marker.*

Figure 5. *Phylogenetic relationship between concatenated sequences of seven housekeeping genes of sequence types (STs) determined for E. coli isolates based on the neighbour-joining analysis.*

Table 1. Characterization of avian *E. coli* isolates from outbreaks of colibacillosis (post mortem – liver, spleen, heart, pericardium). Multilocus sequence type (ST), serotyping (O, K and H antigens) – all strains were β -glucuronidase positive and haemolysin negative. K+ (possibly an acidic capsule polysaccharide K antigen which can not be identified – precipitate with Cetavlon and the O antigen is neutral); K- (Negative reaction with Cetavlon – no acidic capsule K is present); H- (Non-motile); F11 pili (\pm); Ribotypes using HindIII and EcoRI (arbitrary numerals and letters are assigned for different ribotypes for clinical isolates – plasmids harbored by the strains are stated individually (kb) or collectively as small plasmids (<30 kb); colicin production (V \pm / other \pm).

Outbreak number Number of isolates Sequence Type (ST)	Source Tissue / Age (wks)	Serotype	Ribotypes		Plasmids (Kb)	Colicin V / other \pm / \pm
			EcoRI	HindIII		
Outbreak 1 <i>n</i> =6 <i>ST</i> 95	Spleen (8)	O2:K1:H7	I	A	135 (all six isolates)	- / -
	Spleen (8)	O2:K1:H7	I	A		- / -
	Spleen (8)	O2:K1:H7	I	A		- / -
	Spleen (8)	O2:K1:H7	I	A		- / -
	Spleen (8)	O2:K1:H7	I	A		- / -
	Spleen (8)	O2:K1:H7	I	A		- / -
Outbreak 2 <i>n</i> =6 <i>ST</i> 117	Liver+Spleen (11)	O111:K-:H4	II	B	105; 30 (+ small) (all three isolates)	+ / +
	Liver+Spleen (11)	O111:K-:H4	II	B		+ / +
	Liver+Spleen (11)	O111:K-:H4	II	B		+ / +
Outbreak 3 <i>n</i> =8 <i>ST</i> 117	Spleen+Liver (12)	O111:K-:H4	II	B	120; 30 (+ small) (all four isolates)	+ / +
	Spleen+Liver (12)	O111:K-:H4	II	B		+ / +
	Spleen+Liver (12)	O111:K-:H4	II	B		+ / +
	Spleen+Liver (12)	O111:K-:H4	II	B		+ / +
Outbreak 4 <i>n</i> =8 <i>ST</i> 93	Spleen+Liver (12)	O1:K+:H4	IV	D	120; 70 (+/- small) (all four isolates)	- / -
	Spleen+Liver (12)	O1:K+:H4	IV	D		- / -
	Spleen+Liver (12)	O1:K+:H4	IV	D		- / -
	Spleen+Liver (12)	O1:K+:H4	IV	D		- / +
Outbreak 5 <i>n</i> =4 <i>ST</i> 95	Lung (18)	O78:K-:H-	IV	D	150; 105; 90 (+ small) (all three isolates)	- / -
	Lung+Spleen	O78:K-:H-	IV	D		+ / +
	+Pericardium (18)	O78:K-:H-	IV	D		- / -

Outbreak 6 <i>n=4</i> <i>ST23</i>	Lung (18)	O18ac:K1:H	I	C	120; 30	+ / +
	Spleen (18)	7	I	C	(+ small)	+ / +
	Spleen+Pericardium	O18ac:K1:H	IV	D	(two isolates)	+ / -
		7 O78:K:-H-			135 (one isolate)	
Outbreak 7 <i>n=19</i> <i>ST95</i>	Spleen+Liver (11)	O18ac:K1:H	I	C	120	- / +
	Spleen+Liver (11)	7	I	C	(+ small)	- / +
		O18ac:K1:H			(both isolates)	
		7				
	Spleen+Liver (13)	O18ac:K1:H	I	C	120	- / +
	Spleen+Liver (13)	7	I	C	(both isolates)	- / +
		O18ac:K1:H				
		7				
	Liver (13)	O18ac:K1:H	I	C	120; 30	- / +
	Liver (13)	7	I	C	(both isolates)	- / +
		O18ac:K1:H				
		7				
	Liver+Heart (13)	O18ac:K1:H	III	E	120 (one isolate)	- / +
	Spleen+Liver (13)	7	I	C		- / +
	Spleen+Liver (13)	O18ac:K1:H	I	C	30 (two isolates)	- / +
		7				
		O18ac:K1:H				
		7				
	Liver+Spleen (13)	O18ac:K1:H	I	C	120; 70	- / +
	Liver (13)	7	I	C	(both isolates)	- / +
		O18ac:K1:H				
		7				

Fig. 1a

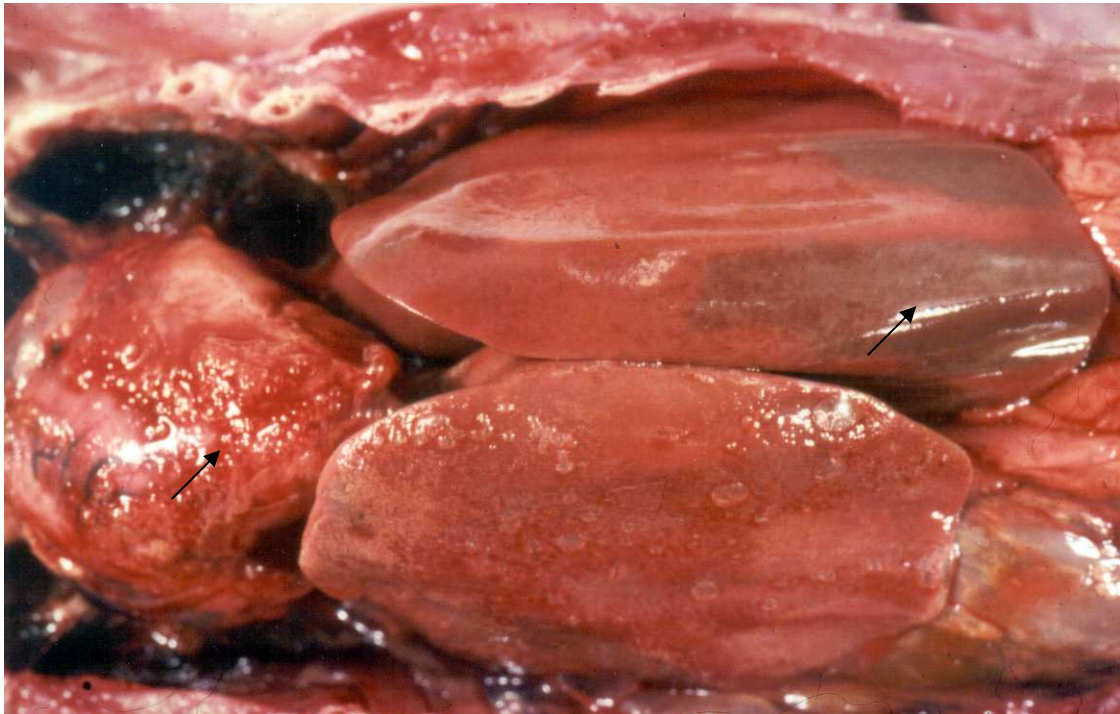


Fig 1b

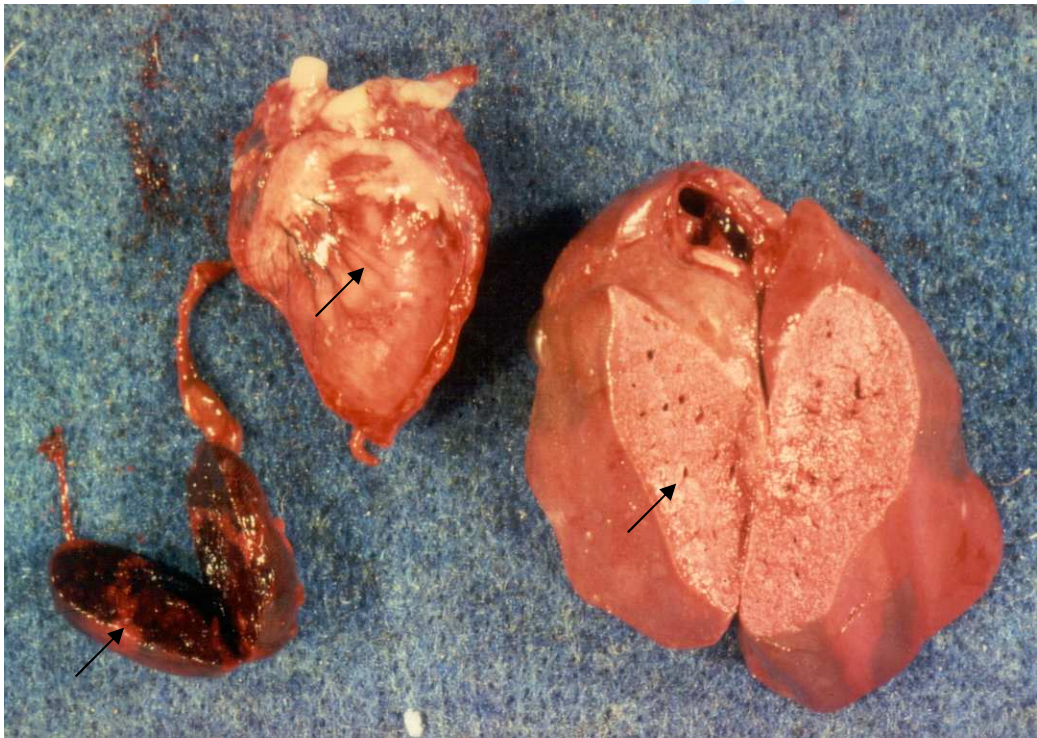


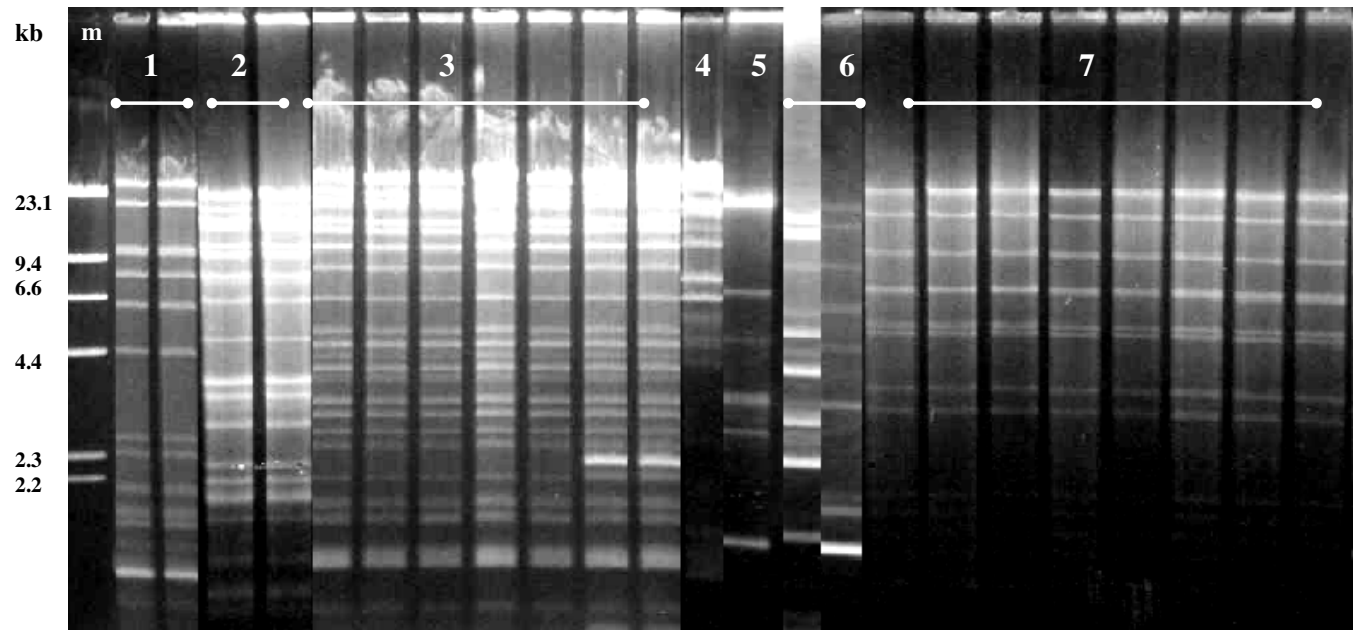
Figure 2.

Figure 3.

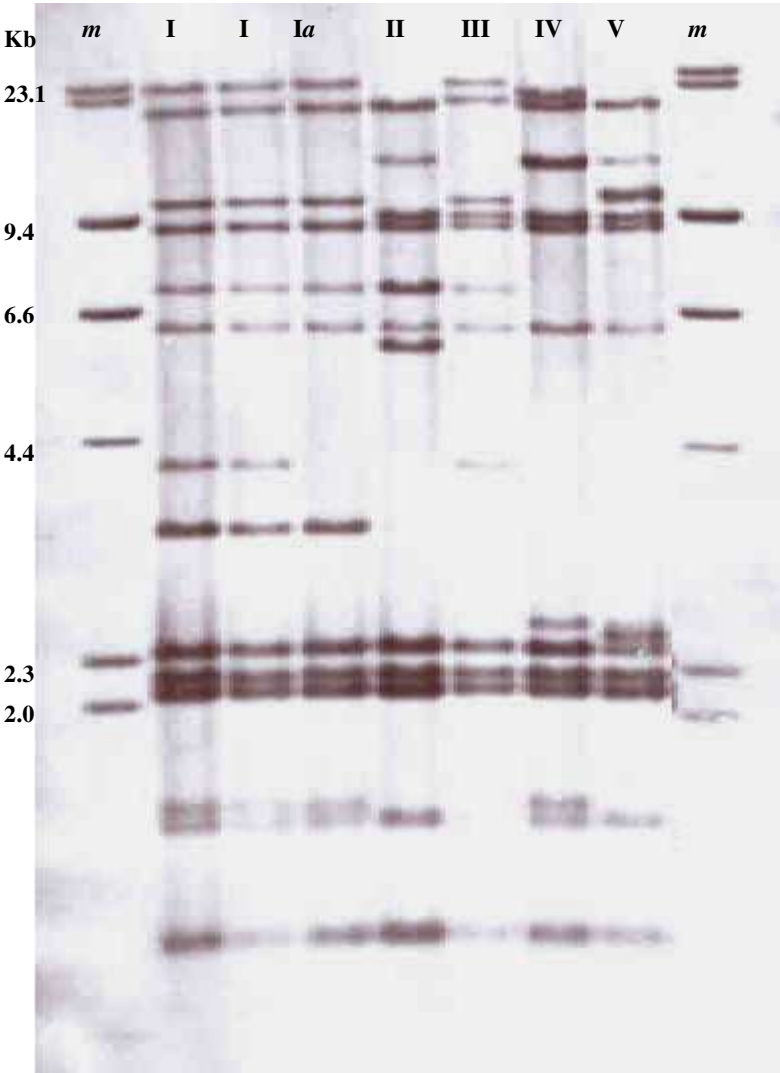


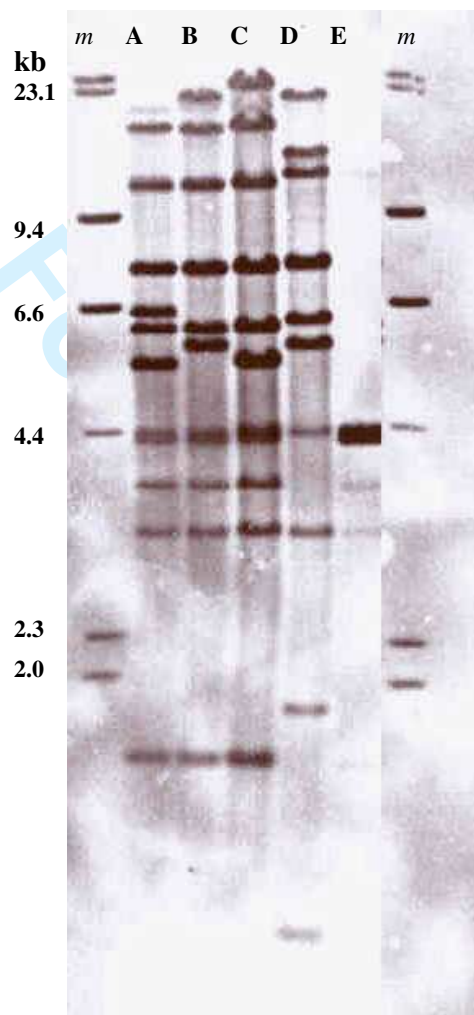
Figure 4.

Fig. 5

