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Sioned Timothy¹, Khaliq Shafi¹, A. Howard Leatherbarrow¹, F.T.W Jordan¹ and Paul Wigley^{2*}

¹Department of Veterinary Pathology and ²National Centre for Zoonosis Research,
University of Liverpool, Leahurst Neston CH64 0SH, United Kingdom

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* Corresponding author

Tel 44 (0) 151 7946193

Fax 44 (0) 151 7946005

Email: paul.wigley@liv.ac.uk

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Abstract

The molecular epidemiology of 70 *Escherichia coli* isolates from an infection outbreak in a layer breeder flock was examined by pulsed field gel electrophoresis (PFGE) and for a range of virulence factors by PCR. PFGE showed 35 of 45 isolates from eight disease cases was associated with a single clonal group that was the exclusive strain associated with reproductive tract. A second unrelated group was found in environmental isolates and healthy birds. Remaining isolates were unrelated to each other or either clonal group. PCR virulotyping indicated the 'epidemic' clonal group contains virulence factors including *iss*, *sfa*, *tsh*, *iucC*, *ibeA*, and *sitA* associated with avian pathogenic *E. coli* plus several virulence factors more normally associated with human urinary tract infection. Significantly the 'epidemic' clone was also found in an environmental sample suggesting it may have been transmitted to the flock via the environment.

Introduction

Avian pathogenic *Escherichia coli* (APEC) cause extra-intestinal disease and are a major pathogen of poultry production (La Ragione *et al.*, 2002; La Ragione & Woodward, 2002). Colibacillosis is usually associated with broiler production, but recently cases in laying hens have been described (Vandekerchove *et al.*, 2004; Vandekerchove *et al.*, 2005). Several serotypes cause colibacilloiosis and a range of virulence factors have been described (Dho-Moulin & Fairbrother, 1999; Ewers *et al.*, 2005; La Ragione & Woodward, 2002; McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005). The molecular epidemiology of APEC outbreaks have been investigated by PCR-based methods particularly for the presence of a range of virulence factor genes and pulsed field gel electrophoresis (PFGE) which has proved a reliable method (Ewers *et al.*, 2004; Jeffrey *et al.*, 2004).

An outbreak of colibacillosis associated with reproductive tract infection, salpingitis and peritonitis was described in a layer breeder flock (Jordan *et al.*, 2005). Here we begin to determine the molecular epidemiology of the outbreak and characterise the *E. coli* associated with diseased and healthy birds along with the environment of the poultry house through PFGE and PCR virulotyping.

Materials & Methods

Bacterial strains. Seventy *E. coli* isolates were collected in March and October 2002 (Jordan *et al.*, 2005), including 45 isolates from 8 chickens that had died as a result of infection during the laying period 16 isolates from the gastrointestinal tract of three healthy chickens and nine isolates from feed, water and litter. Isolates stored at -70 °C were reconstituted on nutrient agar and maintained on agar slopes for the duration of the study.

Pulsed-field Gel Electrophoresis. Block preparation and PFGE were performed according to the Standardised PulseNet Rapid *E. coli* PFGE method (http://www.cdc.gov/PULSENET/protocols/ecoli_salmonella_shigella_protocols.pdf) with slight modifications as previously described (Hughes *et al.*, 2008). PFGE banding patterns were analysed according to PulseNet Europe protocols (<http://www.pulsenet-europe.org>) through the 'Bionumerics' programme (Applied Maths, Belgium). Matching and dendrogram UPGMA (unweighted pair groups method with averages) of the banding patterns were performed using the Dice coefficient with a 2% tolerance window.

PCR Virulotyping. Details of target genes, gene function and primer sequences are given in Table 1. Bacteria were grown overnight on nutrient agar at 37°C. DNA was extracted by boiling for 10 minutes. Amplification was performed in a 25 µl reaction mixture containing 1 µl of DNA extract, 1 µl of each primer and 23 µl of PCR mastermix (Abgene, Surrey). PCR conditions were based on those of Johnston & Stell (2000). APEC strain 421/95 was used as a control.

Results and Discussion

All but one clinical case was found to harbour *E. coli* from a single clonal group within its reproductive tract. PFGE analysis indicated that 35 of the 45 isolates from clinical cases, including all but one reproductive tract isolate, clustered into this group with excess of 90% similarity (Figure1). One environmental (litter) isolate also fell into this group indicating that the environment is a potential source of the outbreak. A second group, we term the 'environmental clone', with over 95% identity between isolates was found in the environment and healthy birds. Remaining isolates formed distinct individual patterns unrelated to each other or either clonal group (<75% similarity).

Other isolates from clinical cases were from the large intestine and appeared unrelated to each other, suggesting these represent part of the normal flora.

A marked distinction between isolates recovered from colibacillosis lesions and from the environment with respect to serotype and PFGE type has been shown (Jeffrey *et al.*, 2004). A similar association was noted in this study. Extensive PFGE-based surveys of APEC isolates in Germany over a 12 year period also indicate that a limited range of large clonal groups are associated with disease in chickens (Ewers *et al.*, 2004). However identification of APEC by PFGE pattern alone is not possible.

Isolates of the 'epidemic clone' from seven different birds, four isolates of the 'environmental clone', three other environmental isolates and the VLA strain 421/95 were selected for PCR virulotyping. Additional positive controls representing various capsule types of *E. coli* were obtained for capsule genes. Results are summarised in Table 2. As expected each isolate from the clonal groups gave the same virulotype as other isolates within the group. Virulotyping revealed that the 'epidemic clone' contained a number of virulence factors which have been described as prevalent in APEC strains (Germon *et al.*, 2005; Ewers *et al.*, 2007; Rodriguez-Siek *et al.*, 2005). These include *iss* found in 80-83% of APEC isolates, *ibeA* found in around 15% of APEC (Germon *et al.*, 2005), *tsh* found in 53-63% of APEC, *iucC* found in over 75% of APEC, *sitA* found in over 85% of APEC (Germon *et al.*, 2005; Ewers *et al.*, 2007; Rodriguez-Siek *et al.*, 2005). Virulence factors more common in human urinary tract pathogenic *E. coli* (UPEC) including *sfa* and the capsule biosynthesis genes *kpsMT* (K1) and *kpsMTII* were also present in the 'epidemic clone' (Rodriguez-Siek *et al.*, 2005). In contrast the 'environmental clone' was positive for only *iss* and *tsh* and other isolates from the environment or healthy birds had few APEC-associated virulence factors. The single clinical isolate not associated with the epidemic clone was positive for only *sitA* suggesting this isolate may be a commensal that contaminated tissues at post mortem examination.

The 'epidemic' clonal group associated with infection contained several well described APEC virulence factors and these were less frequent in environmental and commensal isolates. Several genes found rarely in APEC but frequently in UPEC were also present in the 'epidemic' clonal group. Recently it has been suggested that APEC have a close relationship to human extra-intestinal *E. coli* such as UPEC (Ewers *et al.*, 2007; Johnson *et al.*, 2007; Rodriguez-Siek *et al.*, 2005). The K1 capsular antigen and S-type fimbriae (*sfa*) are frequently found in UPEC but several other virulence factors are common to APEC and UPEC. It is tempting to speculate that virulence factors involved in infection of the mammalian urinary tract would also be advantageous in colonisation of the avian reproductive tract particularly the oviduct, though this would require considerable additional work to establish such a phenotype. In conclusion we show that an outbreak of *E. coli* infection in a layer breeder flock was associated with a single capsular clonal group containing a range of virulence factors both typical and atypical of APEC. The presence of the clonal group in a litter sample suggests the possibility of an environmental source.

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Figure Legends

Figure 1

PFGE analysis following XbaI digestion and generated dendrogram illustrating clonal relationships of 13 E. coli isolates:

Lanes 1-4: isolates obtained from clinically healthy birds; lanes 5-7: environmental isolates. Lanes 4-7 show examples of the 'environmental clonal group' found in environmental samples and healthy birds. Examples of the 'epidemic clonal group' from clinical case are shown in lanes 8-13 of the figure

Table 1 *APEC virulence genes, gene product function and primer sequences used in PCR virulotyping*

Gene	Gene product function	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>sfa</i>	S type fimbriae	F ctccggagaactgggtgcatcttac R cggaggagtaattacaaacctggca	410	Johnson & Stell (2000)
<i>iss</i>	Increased serum survival	F gtggcgaaaactagtaaaacagc R cgcctcgggggtggataa	762	Tivendale et al 2004
<i>tsh</i>	Temperature sensitive haemagglutinin/ autotransporter	F ggtggtgcactggagtgg R agtccagcgtgatatgtg	642	Tivendale et al 2004
<i>kpsMT II</i>	K1 & K5 capsule	F gcgcatttgctgatactgttg R catccagacgataagcatgagca	272	Johnson & Stell (2000)
<i>kpsMT III</i>	K54 capsule	F tcctcttgctactattccccct R aggcgtatccatccctcctaac	392	Johnson & Stell (2000)
<i>kpsMT K1</i>	K1 capsule	F tagcaaactgttctattggtgc R catccagacgataagcatgagca	153	Johnson & Stell (2000)
<i>iucC</i>	Aerobactin synthesis	F cgccgtggctggggtgaag R cagccgggtcaccaagtatcactg	541	Skyberg et al., 2003
<i>hlyD</i>	Haemolysin transport	F ctccggtacgtgaaaaggac R gccctgattactgaagcctg	904	Rodriguez-Siek et al (2005)
<i>ibeA</i>	Invasion	F aggcaggtgtgcgcccgctac R tgggtctccggcaaaccatgc	171	Johnson & Stell (2000)
<i>sitA</i>	Iron transport	F agggggcacaactgattctcg R taccggggccgttttctgtgc	608	Rodriguez-Siek et al (2005)
<i>papC</i>	pilus	F gtggcagtatgagtaatgaccgtta R atatcctttctgcagggatgcaata	205	Johnson & Stell (2000)

Table 2 . Detection of APEC virulence genes in *E. coli* strains. Results for epidemic and environmental clonal groups were identical for 8 and 5 different isolates respectively. (ND= not done). Non-clonal isolates show less than 75% PFGE similarity of banding pattern to either clonal group or each other

Isolate	Target Gene										
	<i>iss</i>	<i>tsh</i>	<i>ibeA</i>	<i>iucC</i>	<i>hlyD</i>	<i>papC</i>	<i>sitA</i>	<i>sfa</i>	<i>kpsMT II</i>	<i>kpsMT III</i>	<i>kpsMT K1</i>
Control (421/95)	+	-	+	+	-	+	+	+	ND	ND	ND
Epidemic clonal group (>90% PFGE similarity)	+	+	+	+	-	-	+	+	+	-	+
Environmental clonal group (>95% PFGE similarity)	+	+	-	-	-	-	-	-	-	-	-
Clinical case non-clonal	-	-	-	-	-	-	+	-	-	-	-
Commensal isolate (non-clonal)	-		+	-	-	-	+	-	-	-	-
Feed isolate (non clonal)	-	+	-	-		+	-	-	+	-	-

