Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model

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Avian pathogenic *Escherichia coli* (APEC) and uropathogenic *E. coli* (UPEC) establish infections in extraintestinal habitats of different hosts. As the diversity, epidemiological sources and evolutionary origins of extraintestinal pathogenic *E. coli* (ExPEC) are so far only partially defined, in the present study, 100 APEC isolates and 202 UPEC isolates were compared by their content of virulence genes and phylogenetic groups. The two groups showed substantial overlap in terms of their serogroups, phylogenetic groups and virulence genotypes, including their possession of certain genes associated with large transmissible plasmids of APEC. In a chicken challenge model, both UPEC U17 and APEC E058 had similar LD₅₀, demonstrating that UPEC U17 had the potential to cause significant disease in poultry. To gain further information about the similarities between UPEC and APEC, the *in vivo* expression of 152 specific genes of UPEC U17 and APEC E058 in both a murine urinary tract infection (UTI) model and a chicken challenge model was compared with that of these strains grown statically to exponential phase in rich medium. It was found that in the same model (murine UTI or chicken challenge), various genes of UPEC U17 and APEC E058 showed a similar tendency of expression. Several iron-related genes were upregulated in the UTI model and/or chicken challenge model, indicating that iron acquisition is important for *E. coli* to survive in blood or the urinary tract. Based on these results, the potential for APEC to act as human UPEC or as a reservoir of virulence genes for UPEC should be considered. Further, this study compared the transcriptional profile of virulence genes among APEC and UPEC *in vivo*.

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INTRODUCTION

Urinary tract infections (UTIs) are among the most common diseases in the world, causing significant morbidity and mortality (Salysers & Whitt, 1994). Each year, approximately 11.3 million community-acquired

UTIs occur in the United States, with an annual cost of \$1.6 billion (Foxman *et al.*, 2000). Most community-acquired UTIs are due to uropathogenic *Escherichia coli* (UPEC) infections (Donnenberg & Welch, 1996; Johnson, 1991). UPEC strains possess specialized virulence factors, enabling them to colonize and invade the host, disrupt the host defence mechanisms, injure host tissues, and/or stimulate a noxious host inflammatory response. The abilities of UPEC to grow extraintestinally may enable them to cause a variety of diseases, not just UTIs.

Avian colibacillosis refers to any localized or systemic infection such as an acute fatal septicaemia or subacute pericarditis and airsacculitis caused entirely or partly by avian pathogenic *Escherichia coli* (APEC) (Barnes *et al.*, 2003). It is responsible for large financial losses to the

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Abbreviations: APEC, avian pathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; qRT-PCR, quantitative real-time reverse transcription-PCR; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

The GEO accession number for the expression of specific genes of UPEC and APEC *in vivo* and *in vitro* is GSE11509.

poultry industry each year due to mortality, lost production and condemnations.

A variety of serotypes have been identified among APEC, and strains of the same O-types (O1, 2, 4, 6, 7, 8, 16, 18 and 75) are also noticeably involved in UTIs (Bonacorsi *et al.*, 2000; Oelschlaeger *et al.*, 2002; Bullen *et al.*, 2005). The common presence of a set of virulence-associated genes among APEC and UPEC strains as well as similar disease patterns and phylogenetic background indicate a genetic relationship between APEC and UPEC isolates (Kaper *et al.*, 2004; Moulin-Schouleur *et al.*, 2006; Ron, 2006). A few studies have investigated the expression *in vivo* of specific genes and the whole transcriptome of UPEC strain CFT073 in a murine urinary infection model, and the upregulation of some genes such as iron-related genes and outer-membrane protein encoding genes was observed (Snyder *et al.*, 2004, 2005; Hagan & Mobley, 2007; Haugen *et al.*, 2007). However, there is little information regarding the comparison of the expression of specific genes between UPEC and APEC isolates during an infection *in vivo*, which is an essential step to define whether APEC can serve as a source of human extraintestinal pathogenic *E. coli* (ExPEC) or as a reservoir of virulence genes for human ExPEC.

In order to define the role of virulence factors during the establishment of infections and the genetic relationship of virulence genes between APEC and UPEC in China, we compared specific virulence genes and their *in vivo* expression between Chinese APEC and UPEC isolates.

METHODS

Bacterial strains. A total of 302 *E. coli* isolates were used in this study, including 202 isolates from humans with community-acquired UTIs, originating from Jiangsu province and Anhui province in eastern China in 2007, and 100 isolates [serogroup: O1 ($n=41$), O2 ($n=31$), and O18 ($n=28$)] implicated in avian colibacillosis that were collected from various regions of China (Song *et al.*, 1999). Enterohaemorrhagic *E. coli* (EHEC) 933 strain was kindly provided by Dr Qiyi Wen in the authors' laboratory. *E. coli* K-12 was purchased from the China Institute of Veterinary Drug Control.

Isolation of UPEC strains was performed according to standard laboratory protocols and UPEC were isolated from $>10^5$ c.f.u. of a uropathogen per millilitre of midstream urine documented in at least two separate cultures obtained at least 24 h apart. Only one isolate from each patient was analysed. After isolation, the bacteria were kept frozen at -70°C after the addition of 20% (v/v) glycerol and they were not subcultured more than twice before the investigation.

Serotyping. Serotyping of UPEC isolates was performed by standard methods at the China Institute of Veterinary Drug Control.

Haemolysis determination. APEC and UPEC isolates were assessed for haemolysis activity on blood agar plates (supplemented with 5% sheep blood) using standard methods (Forbes *et al.*, 1998).

Phylogenetic typing. APEC and UPEC isolates were assigned to phylogenetic groups by multiplex PCR as previously described (Hagberg *et al.*, 1983). The phylogenetic group of each strain was determined as follows: *ChuA* (+), *YjaA* (+), *TSPE4.C2* (+/-) was assigned to group B2; *ChuA* (+), *YjaA* (-), *TSPE4.C2* (+/-) to

group D; *ChuA* (-), *YjaA* (+/-), *TSPE4.C2* (+) to group B1; and *chuA* (-), *YjaA* (+/-), *TSPE4.C2* (-) to group A.

Virulence genotyping. UPEC and APEC strains were investigated for 37 virulence-related genes by multiplex and single PCR assays, as previously described (Johnson & Stell, 2000; Rodriguez-Siek *et al.*, 2005). Targeted genes and their descriptions as well as primer sequences for the amplification procedures are given in Table 1. All primers used in amplification of the virulence genes were obtained from Sangon (Shanghai, China).

The amplified products were visualized by standard gel electrophoresis of 15 μl of the final reaction mixture in 1.0% agarose gels in Tris/acetate/EDTA (TAE) buffer at 5 V cm^{-1} . The gels were stained with ethidium bromide ($0.5\text{ }\mu\text{g ml}^{-1}$) for 2 min, washed in distilled water, analysed under UV light (300 nm) and photographed. DNA Ladder 100 bp was used as molecular size marker. All amplification procedures were repeated three times.

Virulence assay for 1-day-old chicks. The virulence of APEC E058 and UPEC U17 was determined using an LD₅₀ test for 1-day-old chicks (Dho & Lafont, 1984). Briefly, UPEC U17 and APEC E058 were grown to OD₆₀₀ 0.4 in Luria-Bertani (LB) broth, pelleted by centrifugation, and resuspended in PBS to densities of 10^8 , 10^7 , 10^6 , 10^5 and 10^4 c.f.u. ml^{-1} . For each bacterial density, five 1-day-old specific-pathogen-free chicks were inoculated subcutaneously with 0.1 ml of the suspensions. LD₅₀ was calculated by the method of Reed & Muench (1938). EHEC 933 and *E. coli* K-12 were used as controls.

All of the dead birds and the euthanized birds at 48 h post-inoculation underwent necropsy. Macroscopic fibrinous lesions were observed, and isolation of bacteria from the thoracic air sac, pericardial fluid and blood (if available) was performed on MacConkey agar. The isolates were subjected to serogroup typing and virulence genotyping by PCRs.

Chicken challenge model. An *E. coli* infection model using 1-day-old specific-pathogen-free chicks was used. Briefly, 10 chickens (1 day old) were infected with 10^7 c.f.u. of UPEC U17 or APEC E058 by the air sac route. Four hours following challenge with the *E. coli* strains, chickens were euthanized. Blood was collected directly into a 15 ml centrifuge tube containing 1 ml anticoagulant (0.5% sodium citrate). The tube was centrifuged (5 min, 500 g, 4°C), and the bacteria-containing supernatant was collected into another tube. The supernatants were centrifuged (10 min, 2500 g, 4°C), and the bacterial pellet was frozen at -70°C until RNA extraction.

BALB/c mouse UTI model. Forty BALB/c mice were transurethrally inoculated as previously described (Hagberg *et al.*, 1983; Forbes *et al.*, 1998) with 1×10^8 c.f.u. of UPEC U17 or APEC E058. The infection was monitored daily by pooling urine from 10 mice at random and spiral plating on MacConkey agar to quantify c.f.u.. Mice were reinoculated every 2 days to maintain infection at levels between 2×10^6 and 3×10^7 c.f.u. per ml of urine, which allowed the collection of sufficient numbers of bacteria for RNA extraction and subsequent analysis. Urine was collected for 15 days, always allowing at least 18 h between inoculation and urine collection. On average, 50 μl of urine was collected from each mouse every 45 min during an 8 h period. Urine was collected directly into 1.5 ml microcentrifuge tubes containing 650 μl RNA Protect Bacterial Reagent (Qiagen) and was pooled until the total volume reached approximately 1 ml. The RNA Protect Bacterial Reagent-treated sample remained on ice until all samples had been collected for that day. A crystalline precipitate was allowed to settle before pipetting off the bacteria-containing supernatant into a 15 ml centrifuge tube for collection. Tubes were centrifuged (15 min, 2500 g, 4°C), the supernatant was decanted, and the bacterial pellet was frozen at -70°C until RNA extraction.

Table 1. Primers used for the amplification study

Gene	Amplicon size (bp)	Primer sequence	Reference
pTJ100-related genes			
<i>cvaC</i>	679	F cacacacaacgggagctgt R cttccgcagcatagttccat	Johnson & Stell (2000)
<i>iroN</i>	667	F aagtcaaagcaggggtgcccg R gacgccgacattaagacgcag	J. R. Johnson protocols*
<i>iss</i>	323	F cagcaaccgaaccacttgatg R agcattgccagagcggcagaa	J. R. Johnson protocols
<i>iucC</i>	541	F cgccgtggctgggtaag R cagccggttcaccaagtatcactg	Skyberg <i>et al.</i> (2003)
<i>iutA</i>	302	F ggctggacatcatgggaactgg R cgtcgggaacgggtagaatcg	Johnson & Stell (2000)
<i>sitA</i>	608	F agggggcacaaactgattctcg R taccgggccgttttctgtgc	Runyen-Janecky <i>et al.</i> (2003)
<i>traT</i>	290	F ggtgtggtgcgatgagcacag R cacggttcagccatccctgag	Johnson & Stell (2000)
Adhesins			
<i>afa</i>	594	F ggcagagggccggcaacaggc R cccgtaacgcgcagcatctc	Johnson & Stell (2000)
<i>bmaE</i>	507	F atggcgctaacttgccatgctg R agggggacatagcccccttc	Johnson & Stell (2000)
<i>fimH</i>	508	F tcgagaacggataagccgtgg R gcagtcacctgccctccgta	Johnson & Stell (2000)
<i>gafD</i>	952	F tgttggaccgtctcagggtc R tcccggaaactcgtgttact	Johnson & Stell (2000)
<i>iha</i>	829	F ctggcggaggctctgagatca R tccttaagctccgcggctga	J. R. Johnson protocols
<i>papA</i>	717	F atggcagtggtgttttggtg R cgtccaccatacgtgctcttc	Johnson & Stell (2000)
<i>papC</i>	205	F gtggcagtatgagtaataaccgtta R atatccttctgcagggatgaata	Johnson & Stell (2000)
<i>papEF</i>	326	F gcaacagcaacgctggttgcatcat R agagagagccactctatacggaca	Johnson & Stell (2000)
<i>papG</i> allele I	461	F tcgtgctcaggtccggaattt R tggcatcccccaacattatcg	Johnson & Stell (2000)
<i>papG</i> allele I'	479	F ctactatagttcatgtcaggtc R cctgcattcctccaccattatcga	Johnson & Stell (2000)
<i>papG</i> allele II	190	F gggatgagcgggcctttgat R cgggcccccaagtaactcg	Johnson & Stell (2000)
<i>papG</i> allele III	258	F ggcctgcaatggatttacctgg R ccaccaaatgaccatgccagac	Johnson & Stell (2000)
<i>sfa</i>	410	F ctccggagaactgggtgcatcttac R cggaggagtaattacaacctggca	Johnson & Stell (2000)
<i>sfaS</i>	244	F gtggatacgacgattactgtg R ccgccagcattccctgtattc	Johnson & Stell (2000)
<i>focG</i>	364	F cagcacaggcagtgatagca R gaatgtgcctgcccattgct	Johnson & Stell (2000)
Iron-related			
<i>feoB</i>	470	F aattggcgtgcatgaagataactg R agctggcgacctgatagaacaatg	Runyen-Janecky <i>et al.</i> (2003)
<i>fyuA</i>	787	F tgattaaccccgacgggaa R cgcagtaggcacgatgttgta	Johnson & Stell (2000)
<i>ireA</i>	254	F gatgactcagccacgggtaa R ccaggactcacctcacgaat	J. R. Johnson protocols
<i>irp-2</i>	287	F aaggattcgtgttaccggac R tcgtcgggcagcgtttcttct	Janben <i>et al.</i> (2001)

Table 1. cont.

Gene	Amplicon size (bp)	Primer sequence	Reference
Protectins			
<i>kpsMT</i> (K1)	153	F tagcaaacgttctattgtg used with <i>kpsMT</i> II-Reverse	Johnson & Stell (2000)
<i>kpsMTII</i>	272	F gcgcatttgctgatactgtg R catccagacgataagcatgagca	Johnson & Stell (2000)
<i>kpsMTIII</i>	392	F tcctcttgctactattcccct R aggcgtatccatccctcctaac	Johnson & Stell (2000)
<i>rfe</i>	788	F atccatcaggaggaggactgga R aaccataccaaccaatgcgag	Johnson & Stell (2000)
Toxins			
<i>cdtB</i>	430	F gaaaataaatggaacacacatgtccg F'aaagtaaatggaatataaatgtccg R aaatctctgcaatcatccagtta R'aatcaccaagaatcatccagtta	Johnson & Stell (2000)
<i>cnf-1</i>	1105	F atcttatactggatgggatcatctgg R gcagaacgacgttcttcataagtatc	Johnson & Stell (2000)
<i>hlyD</i>	904	F ctccggtacgtgaaaaggac R gccctgattactgaagcctg	J. R. Johnson protocols
Miscellaneous			
<i>fliC</i> (H7)	547	F acgatgcaggcaacttgacg R ggggttgctgtgcagaacc	J. R. Johnson protocols
<i>ibeA</i>	171	F aggcaggtgtgcccgcgtac R tgggtctccggcaaacatgc	Johnson & Stell (2000)
<i>ompT</i>	559	F atctagccgaagaaggagc R cccgggtcatagtgttcac	J. R. Johnson protocols
<i>malX</i> (PAI)	925	F ggacatcctgttacagcgcgca R tcgccaccaatcacagccgaac	Johnson & Stell (2000)
Phylogenetic typing			
<i>chuA</i>	279	F gacgaaccaacggtcaggat R tgccgccagtaccaaagaca	Clermont <i>et al.</i> (2000)
<i>yjaA</i>	211	F tgaagtgtcaggagacgctg R atggagaatgcgttcctaac	Clermont <i>et al.</i> (2000)
<i>TSPE4.C2</i>	152	F gagtaatgtcggggcattca R cgcgccaacaaagtattacg	Clermont <i>et al.</i> (2000)

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Construction of the microarrays. The confirmed PCR products of 152 genes were precipitated with 2-propanol and redissolved in DNA Spotting Solution (CapitalBio Corp., Beijing, China). These 152 genes include known virulence genes originating from APEC and UPEC, some genes encoding antibiotic resistance ($n=95$), and potential virulence genes of APEC obtained previously by selective capture of transcribed sequence and suppression subtractive hybridization methods ($n=57$) (Huan, 2007; Chen, 2006; Chen *et al.*, 2007). Each gene was printed on amino-silanized slides in triplicate to facilitate subsequent statistical analysis. After printing, the slides were baked at 80 °C for 1 h and stored dry at room temperature until use.

Prior to hybridization, the slides were rehydrated over 65 °C water for 10 s, UV cross-linked at 250 mJ cm⁻². The unimmobilized DNAs were washed off with 0.5 % SDS for 15 min at 42 °C and SDS was removed by dipping the slides in anhydrous ethanol for 30 s. The slides were spin-dried at 1000 r.p.m. for 2 min.

RNA isolation and cDNA synthesis. RNA was extracted using an RNeasy Mini kit with 1 h on-column DNase digestion (Qiagen)

according to the RNeasy Mini kit handbook. Multiple RNA preparations of the same sample were pooled prior to cDNA synthesis. Up to 20 µg of RNA was mixed with 750 ng of random hexamers (Invitrogen) for each cDNA synthesis reaction according to a previously described protocol (Rosenow *et al.*, 2001). SuperScript II reverse transcriptase (1500 U; Invitrogen) was added to these reactions, along with First Strand buffer, dithiothreitol and deoxyribonucleotides at concentrations recommended by the manufacturer (Invitrogen). The reactions were incubated at 25 °C for 10 min, 37 °C for 60 min, 42 °C for 60 min and 70 °C for 10 min. cDNA was purified with the PCR Clean-up NucleoSpin Extract kit (Macherey-Nagel).

Labelling and hybridization. The purified cDNA was labelled with Cy5-dCTP or Cy3-dCTP (Amersham Pharmacia Biotech). The hybridization was performed under LifterSlip (Erie Company). Arrays were scanned with a confocal LuxScan scanner (CapitalBio), and the images obtained were analysed with SpotData software (CapitalBio).

Quantitative real-time reverse transcription-PCR (qRT-PCR).

Primers designed to amplify *ompT* of UPEC U17 and APEC E058 were AACATCCGAATACCGTC and GGAAGCCTCCCTCACTACTA, and primers for *gapA* were CATCGTTTCCAACGCATCCT and ACCTTCGATGATGCCGAAGTT (forward and reverse primers, respectively). Thirty nanograms of cDNA and 300 nM (final concentration) each primer were mixed with 12.5 µl 2 × SYBR Green PCR Master Mix (Takara). Assays were performed in triplicate with an ABI Prism model 7300 instrument. All data were normalized to the internal standard *gapA* (encoding glyceraldehyde-3-phosphate dehydrogenase), and melting curve analysis demonstrated that the accumulation of SYBR Green-bound DNA was target gene specific. The standard curve method was used for analysis, and the data were log₂ transformed to obtain fold-change differences between *in vivo* and *in vitro* growth conditions.

Statistical analysis. The significance of prevalences of phylogenetic groups A, B1, B2 and D between UPEC and APEC isolates was analysed by a log-likelihood test. Significance of changes in gene expression was analysed by Student's *t*-test ($P < 0.05$).

RESULTS

Serotyping of UPEC isolates

Serotyping of the 202 uropathogenic strains revealed that 124 (61 %) were O-antigen typable, and the remaining 78 (39 %) were O-nontypable. Most of the typable strains belonged to O-serotypes O1 (35 isolates), O6 (6 isolates), O7 (6 isolates), O15 (11 isolates), O18 (4 isolates), O25 (7 isolates) and O26 (11 isolates); the other 46 O-serotypable strains were distributed into 30 serogroups.

Haemolysis determination in APEC and UPEC isolates

Among the 202 UPEC isolates, 20 (10 %) caused clearing of blood agar around areas of bacterial growth, while none of the 100 APEC isolates haemolysed blood agar.

Phylogenetic typing of APEC and UPEC isolates

The distribution of APEC and UPEC isolates among the four phylogenetic groups is shown in Table 2. Of the 100 APEC isolates, 42 (42 %) fell into group A, 36 (36 %) into B2, 12 (12 %) into D, and 10 (10 %) into B1. Of the 202 UPEC isolates, 86 (42 %) fell into group D, 77 (38 %) into B2, 26 (13 %) into A and 13 (7 %) into B1.

Table 2. Comparison of phylogenetic groups between APEC and UPEC isolates

Group	APEC (<i>n</i> =100)	UPEC (<i>n</i> =202)	<i>P</i> -value*
A	42 (42 %)	26 (13 %)	<0.01
B1	10 (10 %)	13 (7 %)	NS
B2	36 (36 %)	77 (38 %)	NS
D	12 (12 %)	86 (42 %)	<0.01

*NS, Differences not significant.

Virulence genotyping of APEC and UPEC isolates

Table 3 summarizes the content of virulence genes for APEC and UPEC isolates. Almost all isolates (≥ 90 %) possessed *feoB* and *fimH*. All the pTJ100-related genes occurred in the majority of APEC isolates, with *iroN*, *iss*, *iucC*, *iutA*, *sitA* and *traT* occurring in ≥ 71 % of isolates,

Table 3. Prevalence of virulence genes in APEC and UPEC isolates

Gene	UPEC		APEC	
	No. of isolates	Percentage of UPEC	No. of isolates	Percentage of APEC
pTJ100-related				
<i>cvaC</i>	26	13	53	53
<i>iroN</i>	61	30	89	89
<i>iss</i>	107	53	81	81
<i>iucC</i>	75	37	71	71
<i>iutA</i>	167	83	90	90
<i>sitA</i>	119	59	90	90
<i>traT</i>	151	75	74	74
Adhesins				
<i>afa</i>	9	4	6	6
<i>bmaE</i>	19	9	1	1
<i>fimH</i>	186	92	95	95
<i>gafD</i>	0	0	0	0
<i>iha</i>	87	43	1	1
<i>papA</i>	110	54	17	17
<i>papC</i>	110	54	37	37
<i>papEF</i>	120	59	41	41
<i>papG</i> allele I	29	14	9	9
<i>papG</i> allele I'	0	0	0	0
<i>papG</i> allele II	77	38	43	43
<i>papG</i> allele III	17	8	0	0
<i>sfa</i>	99	49	2	2
<i>sfaS</i>	32	16	2	2
<i>focG</i>	23	11	1	1
Iron-related				
<i>feoB</i>	192	95	90	90
<i>fyuA</i>	166	82	69	69
<i>ireA</i>	37	18	82	82
<i>irp-2</i>	160	79	73	73
Protectins				
<i>kpsMT</i> (K1)	91	45	55	55
<i>kpsMTII</i>	135	67	47	47
<i>kpsMTIII</i>	29	14	2	2
<i>rfe</i>	10	5	1	1
Toxins				
<i>cdtB</i>	24	12	8	8
<i>cnf-1</i>	18	9	0	0
<i>hlyD</i>	23	11	0	0
Miscellaneous				
<i>fliC</i> (H7)	32	16	6	6
<i>ibeA</i>	21	10	17	17
<i>ompT</i>	127	63	60	60
<i>malX</i> (PAI)	93	46	19	19

except for *cvaC*, which occurred in 53 % of APEC. Four of the pTJ100-related genes (*traT*, *sitA*, *iutA* and *iss*) also occurred in >50 % of UPEC isolates. Other pTJ100-related genes were also found in UPEC isolates: *iucC* (37 %), *iroN* (30 %) and *cvaC* (13 %).

Also, other genes not linked to pTJ100 were frequently found in both APEC and UPEC isolates. *irp-2* and *fyuA* genes, involved in the yersinabactin operon, and *ompT* occurred in ≥60 % of both APEC and UPEC isolates. Various *pap* elements, *papA*, *papC* and *papEF*, occurred commonly in UPEC isolates (≥54 %), and *papC* and *papEF* occurred in ≥37 % of the APEC also. *kpsMT* (K1) and *kpsMTII*, involved in the synthesis of capsules, occurred in ≥45 % of both the APEC and UPEC isolates.

Serogroups, virulence gene profiles and phylogenetic types of UPEC U17 and APEC E058

APEC E058 belonged to the O2 serogroup and B2 phylogenetic group, while UPEC U17 fell in the B2 phylogenetic group, and was nontypable by the tested standard O sera (Table 4).

Virulence genotyping of UPEC U17 and APEC E058 showed that they shared the similar virulence gene profile, with *cvaC*, *iroN*, *iss*, *iucC*, *iutA*, *sitA*, *traT*, *fimH*, *papA*, *papC*, *papEF*, *papG* alleleII, *feoB*, *fyuA*, *irp-2*, *kpsMT* (K1), *kpsMTII*, *ompT*, *malX* and *ireA* (20/37 genes tested). By contrast, EHEC 933 contained only *iroN*, *iss*, *papA*, *iha*, *sfa*, *feoB*, *ireA*, *kpsMTIII*, *cnf-1* and *ompT* (sharing only six genes, *iroN*, *iss*, *papA*, *feoB*, *ireA* and *ompT*, with UPEC U17) among the 37 genes tested, and *E. coli* K-12 possessed only *traT*, *papA*, *sfa*, *feoB*, *ireA*, *kpsMTIII*, *cnf-1* and *ompT* (sharing only five genes, *traT*, *papA*, *feoB*, *ireA* and *ompT*, with UPEC U17) among the 37 genes tested (Table 4).

Virulence evaluation of UPEC U17 and APEC E058 in chickens

Virulence was evaluated using the LD₅₀ test for UPEC U17 and APEC E058 in chickens. The LD₅₀ of APEC E058 and UPEC U17 in chickens was 10^{4.4} and 10^{3.5}, respectively, while the LD₅₀ of EHEC 933 was 10^{7.1} and *E. coli* K-12 was non-pathogenic to chickens (Table 4). Both UPEC U17 and APEC E058 caused a typical avian colibacillosis, with bacteria invading into air sacs, blood, pericardial fluid, and the typical fibrinous lesions. In addition to the LD₅₀ of EHEC 933 being much higher than those of APEC E058 and UPEC U17, the lesions of chickens inoculated with EHEC 933 were also milder than those caused by APEC E058 and UPEC U17 when chickens were euthanized at 48 h post-inoculation (data not shown). No deaths or lesions were observed in chickens inoculated with *E. coli* K-12. The isolation, serogrouping and virulence genotyping of the bacteria confirmed that the infection of the challenged chickens was caused by the inoculated *E. coli* strains.

Table 4. Comparison of biological characteristics among APEC E058, UPEC U17, EHEC 933 and *E. coli* K-12 isolates

Characteristic	APEC E058	UPEC U17	EHEC 933	K-12
Phylogenetic group	B2	B2	D	A
Serogroup	O2	Nontypable	O157	O28
LD ₅₀	10 ^{4.4}	10 ^{3.5}	10 ^{7.1}	Non-pathogenic
Genes:				
pTJ100-related				
<i>cvaC</i>	+	+	—	—
<i>iroN</i>	+	+	+	—
<i>iss</i>	+	+	+	—
<i>iucC</i>	+	+	—	—
<i>iutA</i>	+	+	—	—
<i>sitA</i>	+	+	—	—
<i>traT</i>	+	+	—	+
Adhesins				
<i>papA</i>	+	+	+	+
<i>papC</i>	+	+	—	—
<i>fimH</i>	+	+	—	—
<i>papEF</i>	+	+	—	—
<i>papG</i> allele II	+	+	—	—
<i>afa</i>	—	—	—	—
<i>bmaE</i>	—	—	—	—
<i>gafD</i>	—	—	—	—
<i>papG</i> allele I	—	—	—	—
<i>papG</i> allele I'	—	—	—	—
Adhesins				
<i>iha</i>	—	—	+	—
<i>papG</i> allele III	—	—	—	—
<i>focG</i>	—	—	—	—
<i>sfaS</i>	—	—	—	—
<i>sfa</i>	+	—	+	+
Iron-related				
<i>feoB</i>	+	+	+	+
<i>ireA</i>	+	+	+	+
<i>fyuA</i>	+	+	—	—
<i>irp-2</i>	+	+	—	—
Protectins				
<i>kpsMT</i> (K1)	+	+	—	—
<i>kpsMTII</i>	+	+	—	—
<i>kpsMTIII</i>	—	—	+	+
<i>rfa</i>	—	—	—	—
Toxins				
<i>cdtB</i>	—	—	—	—
<i>hlyD</i>	—	—	—	—
<i>cnf-1</i>	+	—	+	+
Miscellaneous				
<i>ompT</i>	+	+	+	+
<i>malX</i> (PAI)	+	+	—	—
<i>fliC</i> (H7)	—	+	—	—
<i>ibeA</i>	—	+	—	—

Comparison of expression of virulence genes between UPEC U17 and APEC E058 in the chicken challenge model

DNA microarrays were applied to analyse the transcriptomes of UPEC U17 and APEC E058 grown under both *in vivo* and *in vitro* conditions. The *in vivo*-grown bacterial RNA was obtained from bacteria isolated from infected chickens, and the *in vitro*-grown bacterial RNA was obtained from cultures of UPEC U17 and APEC E058 grown statically to mid-exponential phase at 37 °C in LB. The expression level of each of 152 virulence genes and antibiotic-resistance genes was determined for *in vivo* or *in vitro* conditions. A virulence gene or antibiotic-resistance gene expressed twofold or more than the opposite was considered differentially regulated. All *in vivo*-derived data are described here relative to those of bacteria grown in LB. The GEO accession number for the expression of specific genes of UPEC U17 and APEC E058 in chicken challenge model and *in vitro* is GSE11509.

Table 5 lists the differential expression of 10 genes between UPEC U17 and APEC E058 in the chicken challenge model. Five genes were found to be differentially upregulated and four genes were differentially downregulated during growth *in vivo* for APEC E058. Of these five differentially upregulated genes, two are associated with iron acquisition (*iutA* and *iucCD*), and the other three encode colicin ColV (*cvaC*), a protein involved in capsule synthesis (*neuC*) and protease (*ompT*), respectively. *kpsM*, associated with capsule synthesis, and *tetB*, associated with tetracycline resistance, were almost differentially upregulated. For UPEC U17, three genes were found to be differentially upregulated: *cvaC*, *neuC* and *aec-25* (encoding proline permease II).

Comparison of expression of virulence genes between UPEC U17 and APEC E058 in the murine UTI model

The source for *in vivo*-grown bacteria was the urine from transurethrally infected mice, collected from 1 to 15 days post-inoculation for use in RNA isolation, and the source for *in vitro*-grown bacterial RNA was cultures of UPEC U17 and APEC E058 grown statically to mid-exponential phase at 37 °C in LB. The expression level of each of 152 virulence genes and antibiotic-resistance genes was determined for *in vivo* or *in vitro* conditions. A virulence gene was considered to be differentially regulated if the statistically significant change in expression was greater than twofold. All *in vivo*-derived data are described here relative to growth in LB. The GEO accession number for the expression of specific genes of UPEC U17 and APEC E058 in the murine UTI model and *in vitro* is GSE11509.

Table 6 lists the differential expression of eight genes between UPEC U17 and APEC E058 in the murine UTI model. Four genes were found to be differentially downregulated in the murine UTI model for APEC E058 compared with its expression in LB; these encoded group 2 capsule (*kpsM*), a protein involved in capsule synthesis (*neuC*), an outer-membrane protein (*ompA*), and tetracycline resistance (*tetB*).

For UPEC U17, *tir*, encoding translocated intimin receptor, and *iucCD*, encoding aerobactin protein, were differentially upregulated, while *catI*, encoding chloramphenicol acetyltransferase, and *sta*, encoding heat-stable enterotoxin, were differentially downregulated. *iutA* was almost differentially upregulated (ratio 1.9728) for UPEC U17 *in vivo*.

Table 5. Expression of genes from APEC E058 and UPEC U17 during chicken infection relative to that *in vitro*

Gene	Function	Fold change*	
		UPEC U17	APEC E058
<i>aec-25</i> †	Proline permease II	2.2560 ‡	1.0900
<i>aec-30</i> †	Negative response regulator	0.9597	0.4143 ‡
<i>aes-8</i> §	<i>traT</i> lipoprotein	0.8289	0.4860 ‡
<i>aes-15</i> §	OmpT, protease	1.1349	2.5400 ‡
<i>cvaC</i>	Colicin V	2.9225 ‡	2.8100 ‡
<i>gyrB</i>	DNA gyrase	0.7473	0.4573 ‡
<i>iucCD</i>	Aerobactin protein	1.2056	2.4033 ‡
<i>iutA</i>	Aerobactin receptor	1.4464	7.8400 ‡
<i>mdh</i>	Malate dehydrogenase	0.7195	0.3280 ‡
<i>neuC</i>	Capsular polysaccharides (K antigens)	2.9595 ‡	13.3667 ‡

*Fold change refers to growth *in vivo* relative to growth in LB.

†Putative virulence-associated genes of APEC obtained by selective capture of transcribed sequences.

‡Genes significantly upregulated or downregulated *in vivo* at least twofold are in bold.

§Putative virulence-associated genes of APEC obtained by suppression subtractive hybridization.

Table 6. Expression of genes from APEC E058 and UPEC U17 during murine UTI infection relative to that *in vitro*

Gene	Function	Fold change*	
		UPEC U17	APEC E058
<i>catI</i>	Chloramphenicol acetyltransferase	0.3886 †	0.8413
<i>iucCD</i>	Aerobactin protein	2.0562 †	1.3672
<i>kpsM</i>	Group 2 capsule	0.7064	0.3551 †
<i>neuC</i>	Capsular polysaccharides (K antigens)	0.6004	0.0499 †
<i>ompA</i>	Outer-membrane protein II	0.6744	0.2463 †
<i>sta</i>	Heat-stable enterotoxin	0.4699 †	0.8355
<i>tetB</i>	Tetracycline resistance	0.7405	0.2852 †
<i>tir</i>	Translocated intimin receptor	2.5636 †	1.2142

*Fold change refers to growth *in vivo* relative to growth in LB.

†Genes significantly upregulated or downregulated *in vivo* at least twofold are in bold.

Verification of microarray

qRT-PCR was used to independently verify the level of transcription for a virulence gene, *ompT*, which was found to be upregulated 2.54-fold for APEC E058 in the murine UTI model. *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as the normalizing internal standard. qRT-PCR analysis demonstrated a greater sensitivity than microarray analysis, as *ompT* was upregulated 6.67 ± 0.15 -fold for APEC E058 in the murine UTI model.

DISCUSSION

With increasing knowledge of molecular genetics and pathotypes of ExPEC of human and animal origin, the zoonotic potential of animal-derived strains has become a focus of current research. Several studies have investigated the similarities of UPEC and APEC in their serogroups, virulence genotypes and assignments to phylogenetic groups (Johnson *et al.*, 1998, 2001a, b; Kaper *et al.*, 2004; Mokady *et al.*, 2005; Johnson *et al.*, 2006a; Moulin-Schouleur *et al.*, 2006). It has been proposed that poultry may be a candidate vehicle for *E. coli* capable of causing human urinary tract disease, based on research showing transmission of avian *E. coli* from poultry to humans or similarities between avian *E. coli* and UPEC (Levy *et al.*, 1976; Linton *et al.*, 1977; Ojeniyi, 1989; van den Bogaard *et al.*, 2002). Although previous studies have compared the content of virulence genes between UPEC and APEC, similarities of expression of the specific virulence genes of UPEC and APEC in the same chicken challenge model and/or murine UTI model have not to our knowledge been reported before. In this study, we have compared the genetic similarity and the transcriptional profiles of UPEC and APEC strains collected in China in both a murine UTI model and a chicken challenge model by microarray analysis.

The data presented in this study reveal a considerable overlap between APEC and UPEC isolated in China with

respect to serogroups, assignments to phylogenetic groups, virulence genotypes and pathogenicity evaluation *in vivo*. For instance, serogroups O1 and O18, among the dominant serogroups of UPEC isolates, are also prevalent in APEC (Mokady *et al.*, 2005; Johnson *et al.*, 2006a). Indeed, many serogroups detected among APEC strains have already been implicated in UTIs, suggesting a possible transfer of avian strains to humans or vice versa and thus a potential zoonotic risk of avian strains (Burman *et al.*, 2003).

There was also discernible overlap in APEC and UPEC isolates in their assignments to phylogenetic groups, with some intriguing differences. In agreement with other studies (Clermont *et al.*, 2000; Johnson *et al.*, 2001b), the UPEC isolates used in this study primarily belonged to one of two virulence groups (group B2 or D; Table 2). Although a considerable proportion of APEC strains also fell into group B2 (36%), a higher percentage (42%) belonged to group A. Previous studies have suggested a potential human health threat by avian ExPEC strains, as not just B2 isolates were found to yield comparable virulence gene profiles to human ExPEC but also a large proportion of group A APEC isolates were found to represent a potential zoonotic risk (Johnson & Russo, 2002; Burman *et al.*, 2003).

Results of the gene prevalence studies also revealed some similarities between APEC and UPEC isolates. Virulence genotyping of UPEC U17 and APEC E058 showed that they shared 20 of the 37 virulence genes examined, while the EHEC 933 strain shared only six of these 37 genes with UPEC U17, and *E. coli* K-12 shared only five genes with UPEC U17 (Table 4). Based on these findings, UPEC U17 was more similar to APEC E058 than to EHEC 933 or *E. coli* K-12.

Genes encoding ColV (*cvaC*), salmochelin (*iroN*), serum resistance protein (*iss*), aerobactin (*iucC*), ferric aerobactin receptor (*iutA*), *sit* operon (*sitA*) and transfer protein (*traT*) are known for their frequent or exclusive local-

ization on large transmissible R plasmids, such as pTJ100 in APEC (Johnson & Russo, 2002; Johnson *et al.*, 2006b), and they were clearly associated with APEC strains in our study (Table 3). pTJ100 is a large plasmid known to contain many of the genes associated with APEC virulence, including *iro* and *sit*, the ColV operon, certain genes associated with serum resistance, *iss* and *traT*, and *tsh*. Interestingly, pTJ100 also shows similarities in genetic content to a UPEC plasmid (Johnson *et al.*, 2002). The high prevalence of those pTJ100-related genes, such as *iss*, *iutA*, *sitA* and *traT* (Table 3), and low prevalence of *cvaC*, in UPEC isolates suggested that those genes might reside on non-ColV plasmids or within chromosomal pathogenicity islands rather than on pTJ100-like plasmids (Dobrindt *et al.*, 2001, 2002; Sorsa *et al.*, 2003). Further research will be needed to ascertain the location of these genes in UPEC. In this study, only 10 % of the UPEC isolates examined had haemolytic activity, and only 10 % of these isolates carried the *hlyA* gene. Recently, the *hlyA* gene was found to be overwhelmingly associated with the B2 group of UPEC: 34 % of B2 group strains carried the *hlyA* gene while only 4 % of group A, 5 % of group B1 and 2 % of group D strains possessed this gene (Piatti *et al.*, 2008). In our study, 30 % of B2 group strains (23/77) were *hlyA* positive. The relatively low percentage of haemolytic activity and *hlyA* gene carriage rate (10 %) in our 202 UPEC strains may be partially due to the relatively low percentage of B2 group strains involved in this study. For example, in the study mentioned above, group B2 accounted for 56 % of the isolates collected while this percentage in our study was only 38 % (Table 2).

To compare the similarities of UPEC and APEC in pathogenesis, UPEC isolate U17 and APEC strain E058, with similar virulence gene profiles, were chosen for pathogenicity evaluation in chickens. Although the LD₅₀ of UPEC U17 was slightly lower than that of APEC E058, similar lesions were observed in birds inoculated with either UPEC U17 or APEC E058. As controls, the LD₅₀ of EHEC 933 was 10^{7.1} and *E. coli* K-12 was non-pathogenic to chickens (Table 4). These results imply that the similarity of pathogenicity in chickens was closer between APEC E058 and UPEC U17 than that between APEC E058 and EHEC 933 or *E. coli* K-12, and support the possibility that APEC can serve as a source of human ExPEC or as a reservoir of virulence genes for human ExPEC.

Comparison of the expression of specific virulence-factor genes in both the murine UTI model and the chicken challenge model indicated partial similarity between UPEC and APEC isolates, providing further evidence that APEC potentially serve as a source of UPEC or as a reservoir of virulence genes for UPEC.

The transcriptional analysis of *E. coli* during colonization of the murine urinary tract was feasible because the urinary tract is naturally sterile and because the amount of host nucleic acid contamination from exfoliated epithelial cells is insignificant (Forbes *et al.*, 1998; Mulvey *et al.*, 1998).

This allowed us to collect the urine from experimentally infected mice and directly extract RNAs. To maintain the collected bacteria at levels between 2×10^6 and 3×10^7 c.f.u. per ml of urine for challenged bacteria, we reinoculated the mice every 2 days, which allowed the collection of enough bacteria for RNA extraction and analysis. Urine was not collected within the 18 h after each inoculation, to exclude noncolonizing bacteria present in the inoculum that may have been washed out following transurethral challenge. In another murine UTI model, UPEC CFT073 was reinoculated into the mouse bladder every 6 days (Snyder *et al.*, 2004). In fact, the bacterial recovery from bladders varied between the different UPEC strains even if they were also isolated from the urine of patients with acute pyelonephritis. For these UPEC strains such as *E. coli* 4283, 1682, 3084 and O6K13, the mean c.f.u. of collected bacteria from urine samples of inoculated mice varied from 346 to 17090 (Hagberg *et al.*, 1983). According to this finding and based on preliminary experiments, UPEC U17 was confirmed as a low infection level strain, and needed frequent inoculations to maintain high urine titres.

Microarray data revealed that iron-related genes, *iutA* and *iucCD*, were upregulated in the murine UTI model and the chicken challenge model for both UPEC U17 and APEC E058. Iron acquisition is critical for bacteria to survive in blood or urinary tract, as these environments are iron deprived (Foxman *et al.*, 2000). Knockout of iron-acquisition genes has been shown to attenuate the virulence of many bacteria (Torres *et al.*, 2001; Lawlor *et al.*, 2007). Therefore, iron acquisition from the environment is essential for the pathogenesis of *E. coli*, and our results support this.

We affirm the importance of several known virulence genes by confirming the upregulation of these genes *in vivo*, such as those encoding siderophores, capsules and drug resistances. We have also identified several virulence genes which were downregulated when bacteria were grown *in vivo*, compared with their expression levels *in vitro* (Tables 5 and 6). The mechanism of these downregulated genes is still unknown, and further work is needed to define their biological functions.

In summary, our results suggest that it seems reasonable that UPEC and APEC might show similar adaptations for an extraintestinal lifestyle, which, in turn, enable APEC to cause extraintestinal disease in humans. APEC probably serve as a source or as a reservoir of virulence genes for human ExPEC. Further research will be necessary to determine if APEC can actually overcome the hurdles necessary for transmission to humans via the food chain.

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