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**Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts.**

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**REVIEW ARTICLE**

Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts.

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**REVIEW ARTICLE**

Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts.

**Abstract**

Avian colibacillosis is caused by a group of pathogens designated avian pathogenic *Escherichia coli* (APEC). Despite being known for over a century, avian colibacillosis remains one of the major endemic diseases afflicting the poultry industry worldwide. Autologous bacterins provide limited serotype-specific protection, yet multiple serogroups are associated with disease, especially O1, O2 and O78 among many others. Experimental infection models have facilitated the identification of some key APEC virulence genes and allowed testing of vaccine candidates. Well-recognised virulence factors include; Type 1 (F1) and P (Pap/Prs) fimbriae for colonisation, IbeA for invasion, iron acquisition systems, TraT and Iss for serum survival, K and O antigens for anti-phagocytic activity, and a temperature-sensitive haemagglutinin of imprecise function. Intriguingly, these factors do not occur universally among APEC suggesting the presence of multiple alternative mechanisms mediating pathogenicity. The recent availability of the first complete APEC genome sequence can be expected to accelerate the identification of bacterial genes expressed during infection and required for virulence. High-throughput molecular approaches like signature-tagged transposon mutagenesis (STM) have already proved invaluable in revealing portfolios of genes expressed by pathogenic bacteria during infection and this has enabled identification of APEC O2 factors required for septicaemia in the chicken model. Complimentary approaches, such as *in vivo*-induced antigen technology (IVIAT), exist to define the activities of APEC *in vivo*. In recent years, reverse vaccinology and immuno-proteomic approaches have also enabled identification of novel vaccine candidates in other bacterial pathogens. Collectively, such information provides the basis for the development or improvement of strategies to control APEC infections in the food-producing avian species.

## Introduction

The association of *Escherichia coli* strains with disease conditions in avian species was recognized over a century ago (cited by Sojka & Carnaghan, 1961), but these strains were never accorded a special status. Today, *E. coli* strains causing systemic disease in poultry (avian colibacillosis) are termed avian pathogenic *E. coli* (APEC). Colibacillosis is a disease of severe economic significance to all poultry producers worldwide and is characterised by a diverse array of lesions. Recent reports in Western Europe implicate a resurgence of this disease in the poultry industry, particularly in chicken layers (Zanella *et al.*, 2000; Vandekerchove *et al.*, 2004; Jordan *et al.*, 2005). Depending on the virulence status of the strain, host status and presence and type of predisposing factors, the infection manifests as an initial septicaemia that is followed by either sudden death or localised inflammation in multiple organs. The most common lesions associated with colibacillosis are perihepatitis, airsacculitis and pericarditis, though other syndromes such as egg peritonitis, salpingitis, coligranuloma, omphlitis, cellulitis and osteomyelitis/arthritis may be encountered (reviewed in Barnes & Gross, 1997). In some instances, APEC has been associated with peculiar diseases in specific avian species. In chickens, swollen head syndrome often results from synergistic infection of Turkey rhinotracheitis virus (TRTV) and *E. coli* (Picault *et al.*, 1987; Stehling *et al.*, 2003; Morley & Thompson, 1984). TRTV is believed to cause initial acute rhinitis that is followed by invasion of the facial subcutaneous tissues by *E. coli* (Picault *et al.*, 1987). In turkeys, APEC also causes osteomyelitis complex (TOC) characterised by lesions including green discoloration of the liver, arthritis/synovitis, soft-tissue abscesses, and osteomyelitis of the proximal tibia in an otherwise normal-appearing processed turkey carcass (Huff *et al.*, 2000). Typically, this disease is observed in male adolescent turkeys that have low levels of cell-mediated immunity (Bayyari *et al.*, 1997; Huff *et al.*, 2000), implying that defects in the immunity of individual male turkeys lead to disease.

The natural route of infection for APEC is not clearly defined, though the oral and respiratory routes seem to be significant modes of entry. An estimated 10-15% of avian alimentary tract coliforms have been reported to belong to potentially pathogenic APEC serotypes (Harry & Hemsley, 1965). And consistently, both virulent and avirulent *E. coli* have been shown to colonise and persist efficiently in the intestinal tract with extra-intestinal translocation occurring only in the presence of stressors (Dominick & Jensen, 1984; Leitner & Heller, 1992). The implications of the presence of both pathogenic and commensal strains in the intestinal environment are not clearly discernible though it can be speculated that this could be a leading source of a diverse range of APEC strains as reported for atypical enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (Hornitzky *et al.*, 2005). Strikingly, the intestinal location of APEC provides an excellent opportunity for dissemination into the environment and transmission via faeces. APEC has been reported to persist in the dry environment and dust in poultry houses may harbour up to  $10^6$  colony forming units (CFU) of *E. coli* per gram (Harry, 1964). Inhalation of this contaminated dust is believed to lead to systemic APEC infections. Infection of eggs may occur at laying or during formation in the oviduct, often leading to embryo and early chick mortality. In the United Kingdom, salpingo-peritonitis was the most common form of avian colibacillosis observed in laying hens (Jordan *et al.*, 2005), but what influences presentation of this pathology is unknown.

A major predisposing factor for systemic APEC infections is stress, which can be induced by a variety of agents or inappropriate husbandry practices (see Barnes & Gross, 1997). There are numerous other predisposing factors associated with APEC infections (Barnes & Gross, 1997) and these have led to the notion that this disease could result from opportunistic infection, thus underestimating the virulence of APEC. Consistent with this belief, many serotypes of *E. coli* including those believed to be harmlessly carried in the intestinal tract, have been recovered from diseased birds (Blanco *et al.*, 1998; Rodriguez-Siek *et al.*, 2005a; McPeake *et al.*, 2005). Indeed, earlier workers regarded systemic *E. coli* infection as an indicator of physiological stress in chickens (Kendler & Harry, 1967). Furthermore, experimental evidence indicates that extra-intestinal translocation occurs only in the presence of stressors (Leitner & Heller, 1992). This ability to translocate across either the intestinal or respiratory mucosae and disseminate systemically makes APEC also belong to the extra-intestinal pathogen *E. coli* (exPEC) group. The exPEC group includes neonatal-causing meningitis *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC) which predominantly translocate from the intestinal tract.

Our current understanding of the pathogenesis of colibacillosis has been enhanced by the availability of experimental infection models in target hosts. A considerable amount of information on avian colibacillosis now exists and the molecular virulence determinants of APEC are slowly being unravelled. The recent availability of an APEC O1:K1:H7 complete genome sequence (Johnson *et al.*, 2007a) provides a turning point in our understanding of APEC and also an appropriate launching pad for the application of genetic methods to define the APEC pathotype and virulence. Novel approaches to survey the repertoire, sequence, expression and role in virulence of APEC genes in controlled infection models provide an excellent opportunity to improve our knowledge on APEC virulence mechanisms. Here, we re-examine the APEC pathotype and progress towards unravelling APEC virulence factors mediating carriage and systemic translocation in their natural hosts.

### The APEC pathotype

Unlike other pathogenic groups of *E. coli*, no single trait or group of traits defines the APEC pathotype. Some of the phenotypic and genotypic characteristics associated with this group of pathogens are reviewed below.

**Phenotype.** Biotyping and serotyping are often undertaken on isolates recovered from cases of colibacillosis. In most countries, O1, O2 and O78 represent major *E. coli* serogroups isolated from diseased birds (Sojka & Carnaghan, 1961; Chevillat & Arp, 1978; Cloud *et al.*, 1985; Whittam & Wilson, 1988; Dozois *et al.*, 1992; Ewers *et al.*, 2004). Consequently, representative strains from these serotypes provide the focus for unravelling APEC virulence mechanisms and for the development and evaluation of vaccine candidates. These predominant serogroups can also be recovered from faeces of apparently healthy birds (Blanco *et al.*, 1998), reinforcing the notion that the intestinal tract may be an important natural reservoir for APEC and that predisposing factors may be required to produce disease. Significantly, several studies have reported shared characteristics including serogroups between commensal *E. coli* and APEC (Heller & Drabkin, 1977; Blanco *et al.*, 1998; Rodriguez-Siek *et al.*, 2005a; McPeake *et al.*, 2005), implying that APEC could arise from commensal *E. coli* following acquisition of pathogenic traits. Indeed, widespread variation in phenotypic

and virulence characteristics within a single APEC serotype has been reported. Outer membrane protein profiles, virulence and multi-locus enzyme electrophoresis profiles within a serotype were some of observed variable characteristics (Whittam & Wilson, 1988; Kapur *et al.*, 1992; Chaffer *et al.*, 1999). Despite the variable phenotype, tests for motility, haemolysis and lactose fermentation are sometimes tested in conjunction with serotyping, colicin V and aerobactin production, complement resistance and embryo lethality tests to characterise APEC isolates.

**Genotype.** *Putative virulence gene surveys*

Plasmid-mediated horizontal gene transfer results in extensive diversity within the APEC. APEC plasmids carry several putative virulence factors, though some of these have been encountered in *E. coli* isolates from apparently healthy birds (McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a; Kawano *et al.*, 2006). A high degree of variability in the number, size and virulence traits carried on large plasmids exists in both APEC and isolates from apparently healthy birds (Doetkott *et al.*, 1996). But a recent study (Johnson *et al.*, 2007b) failed to detect APEC-specific plasmid replicons and colicin-related genes in commensal strains. Undoubtedly, APEC plasmids carry a significant number of virulence genes which contribute to the present definition of the APEC genotype. Notably, a 94kb region of a 180kb ColV plasmid carries genes encoding for iron acquisition and transport systems and these are more prevalent in APEC than AFEC (Johnson *et al.*, 2006). Importantly, a link between APEC virulence and possession of ColV plasmids has been revealed by several studies (Ginns *et al.*, 2000; Johnson *et al.*, 2002; Gibbs *et al.*, 2003; Tivendale *et al.*, 2004). Transformation of an avirulent wild type *E. coli* strain with a recombinant plasmid (pHK11) encoding for colicin V led to increased colonisation of the chicken trachea (Wooley *et al.*, 1998). Recently, transferring 2 APEC plasmids (ColV and large R) into a commensal *E. coli* strain by conjugation enhanced its abilities to kill chicken embryos, grow in human urine and colonise the murine kidney (Skyberg *et al.*, 2006). Based on a selection of common traits that include plasmid-borne traits, 2 multiplex PCR protocols have recently been described for the identification of APEC (Skyberg *et al.*, 2003; Ewers *et al.*, 2005). Strains from diseased birds were classified as APEC if they harboured at least 4 of the 8 genes included in the study; P fimbriae (*papC*), aerobactin (*iucD*), iron repressible protein (*irp2*), temperature-sensitive haemagglutinin (*tsh*), vacuolating autotransporter protein (*vat*), enteroaggregative toxin (*astA*), increased serum survival protein (*iss*) and colicin plasmid operon genes (*cva/cvi*), whilst non-pathogenic isolates (as evidenced by chicken infection studies) possessed either none or at most three of the genes (Ewers *et al.*, 2005). Recently, four multiplex PCRs have been developed for virulence genotyping and for establishing a phylogenetic link between avian and human exPEC strains (Ewers *et al.*, 2007). Surprisingly, there is lack of detailed information on the combinations of genes essential for inducing systemic APEC infections. But a previous study suggested that these combinations; Tsh/Pil/Iuc, Tsh/Pap/Iuc pathotypes and Tsh and Iuc virulence-associated markers were important factors for APEC (Ngeleka *et al.*, 2002). A limited association between the virulence gene pattern and the serogroup of APEC strains exists (Ewers *et al.*, 2004). Different associations of virulence genes could reflect the existence of sub-pathotypes or different pathotypes within the present APEC group (Delicato *et al.*, 2003) hence different virulence mechanisms might be employed by the different putative sub-pathotypes. Clearly, the definition of APEC merits further revision.

*Comparative genomics.* Efforts to understand the genetic basis of APEC virulence have been facilitated by comparative genomic studies involving strains from diseased birds with their counterparts from apparently healthy birds or laboratory-adapted strains and also through molecular typing techniques. Genomic suppression subtractive hybridisation (GSSH) has enabled identification of several important APEC virulence factors absent in non-pathogenic *E. coli* (Brown & Curtiss III, 1996; Stocki *et al.*, 2002; Schouler *et al.*, 2004; Mokady *et al.*, 2005; Kariyawasam *et al.*, 2006b; 2007). Independent application of the same approach in different O2 APEC strains led to identification of specific but not identical sequences (Stocki *et al.*, 2002; Schouler *et al.*, 2004) suggesting the presence of a variety of potential virulence factors. These findings were unusual since a high degree of genetic diversity exists between APEC serogroups (Mokady *et al.*, 2005) and as well as within a serogroup (White *et al.*, 1993a).

Evidence linking some APEC clones with human exPEC strains, particularly UPEC and NMEC is being uncovered. Comparative analyses between APEC and human exPEC have revealed striking similarities in genomic islands, virulence genes, overlapping O serogroups and phylogeny (Stordeur *et al.*, 2002; Schouler *et al.*, 2004; Germon *et al.*, 2005; Rodriguez-Siek *et al.*, 2005b; Ron, 2006; Chouikha *et al.*, 2006; Johnson *et al.*, 2007a; Ewers *et al.*, 2007; Kariyawasam *et al.*, 2007). Furthermore, earlier work had shown APEC strains to be easily transmitted to humans (Linton *et al.*, 1977; Ojeniyi, 1989). Indeed, studies have shown that some APEC strains could belong to the same clones as human exPEC strains (Achtman *et al.*, 1986; White *et al.*, 1993b). Recently, it has been reported that very closely related clones of serotype O18:K1:H7 could be recovered from extra-intestinal infections in humans and chickens and that isolates from both species were virulent for chicks (Moulin-Schouleur *et al.*, 2006). Consistent with these observations, whole genome sequence analysis has revealed a high degree of similarity between APEC and exPEC, with only 4.5% of the APEC O1:K1:H7 genome not found in three exPEC genomes (Johnson *et al.*, 2007a). Furthermore, analysis of a diverse collection of APEC, UPEC and NMEC strains suggested that poultry may serve as a vehicle or even reservoir for human exPEC strains and that APEC may be the source of virulence associated genes for exPEC strains (Ewers *et al.*, 2007), suggesting the possibility of a food-borne link between these pathogroups.

Molecular typing methods have also been employed to study APEC, but none have revealed an APEC-specific genotype. Extensive genetic diversity among APEC and within a serotype has been demonstrated by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) typing (Carvalho de Moura *et al.*, 2001) and Random Amplification of Polymorphic DNA (RAPD; Maurer *et al.*, 1998). Predominant serotypes associated with colibacillosis appear phylogenetically distant by multilocus enzyme electrophoresis (White *et al.*, 1993a), though pathogenic clones are relatively closely-related when compared with commensal strains (da Silveira *et al.*, 2003). Multi-locus sequence typing (MLST) of O78 strains has revealed closely related clones to reside in different hosts and a strong correlation between virulence and clonal origin (Adiri *et al.*, 2003). PCR-based phylotyping and MLST have also revealed a link between APEC and human exPEC (Moulin-Schouleur *et al.*, 2007), further suggesting the potential food-borne source of human exPEC.

### 3. Progress towards unravelling APEC virulence factors



**3.1. Colonisation factors.** Early work established the respiratory tract to be a significant route of entry into the host (Gross, 1961), whilst the intestinal tract has been reported to be a reservoir for both pathogenic and non-pathogenic strains (Harry & Hemsley, 1965). Colonisation of multiple internal organs occurs in birds that survive initial septicaemia. In this respect, expression of several adhesins by APEC could enhance preferential colonisation of different sites during the infection process including intestinal carriage (Stordeur *et al.*, 2002).

**3.1.1. Fimbrial adhesins.** Fimbriae are proteinaceous filaments or appendages expressed on the bacterial surface that are believed to mediate adherence to host cells and these consist of different types. Mannose-binding type 1 (F1) fimbriae are commonly encountered in APEC, with frequencies ranging between 70 and 100% (Janssen *et al.*, 2001; Dho-Moulin & Fairbrother, 1999). The role of these fimbriae in colonisation has partly been confirmed using tracheal explants (La Ragione *et al.*, 2000a) and intestinal explants where they target follicle-associated epithelium (Edelman *et al.*, 2003). Although expression of these fimbriae has been demonstrated *in vivo* following experimental infection (Dozois *et al.*, 1994; Pourbakhsh *et al.*, 1997a; 1997b), their role in virulence has remained questionable. A defined *fim* mutant colonised the trachea, lungs and internal organs of germ-free chickens to the same extent as the parent strain (Marc *et al.*, 1998). Conversely, a defined *fimH* mutant lacking adherence properties *in vitro* on chicken pharyngeal and tracheal epithelial cells actually exhibited an increased adherence to tracheal mucosa *in vivo* (Arne *et al.*, 2000).

Besides well-characterised Type I fimbriae, P (Pap/Prs) fimbrial adhesins have been reported to be expressed by bacteria that colonize internal organs but not the trachea (Pourbakhsh *et al.*, 1997b) implying that they may be required for systemic translocation. However, a survey by PCR revealed that 76% of the 1601 avian *E. coli* isolates lacked a *pap* gene cluster that encodes for these fimbriae (Stordeur *et al.*, 2002). Among the *pap*-negative strains were *f17* and/or *afa-8* positive strains, adhesins commonly found in pathogenic *E. coli* of mammals (Bouguenec & Bertin, 1999). Strains harbouring the *f17* gene cluster were lethal for day-old chicks and induced classical colibacillosis lesions indicating that P fimbriae may not be essential for pathogenesis (Stordeur *et al.*, 2004) and that their function may be substituted by other adhesins.

Several other types of fimbriae have been reported in APEC. Avian *E. coli* 1 (AC/1) fimbriae of the S- fimbrial adhesin family (Babai *et al.*, 1997; 2000) have been reported to mediate adherence to avian epithelial tissues *in vitro* and *in vivo* (Yerushalmi *et al.*, 1990). Type IV pili encoded on a conjugative plasmid pO78V have recently been described but their role in pathogenesis remains unclear (Gophna *et al.*, 2003). Additionally, a number of putative adhesins were recently identified by selective capture of transcribed sequences (SCOTS) induced *in vivo* (Dozois *et al.*, 2003) including the recently named Stg fimbriae (Lymberopoulos *et al.*, 2006). It is now clear that Stg fimbriae resemble previously described long polar fimbriae in APEC; both are located in between conserved genes *glmS* and *pstS* and contribute to adherence to epithelial cells (Ideses *et al.*, 2005a; Lymberopoulos *et al.*, 2006). These fimbriae enable adherence of a non-fimbriated *E. coli* K-12 strain to avian lung sections and contribute to colonisation of airsacs but not lungs and trachea (Lymberopoulos *et al.*, 2006).

Other pili-related factors identified by SCOTS include *pilN* and *pilQ* which are believed to encode for plasmid-borne type IV pili. Recently, an *E. coli* common

pilus (ECP) that occurs at high frequencies in most pathogenic *E. coli* including APEC, has been reported to be a universal adhesin mediating epithelial cell colonisation (Rendon *et al.*, 2007).

**3.1.2. Afimbrial adhesins.** Though commonly associated with pathogenic *E. coli* strains from mammals, the *afa-8* gene cluster has been detected amongst several of the *pap*-negative avian strains (Stordeur *et al.*, 2002). The *afa-8* operon encodes for an afimbrial adhesin that contributes to virulence for day-old chicks and induction of classical colibacillosis in a manner similar to *fl7* or *pap*-positive strains (Stordeur *et al.*, 2004). In addition to this well-characterised adhesin, several other putative colonisation factors have been described. Curli are coiled surface structures found in most *E. coli* strains whose role in colonisation has been deduced from their ability to bind fibronectin (Olsen *et al.*, 1989), adhere to chick explant tissues (La Ragione *et al.*, 2000a) and their contribution to persistence in day-old chicks (La Ragione *et al.*, 2000b). An autotransporter temperature-sensitive haemagglutinin (Tsh) has been suggested to contribute to early stages of infection including colonisation of airsacs but not subsequent generalised infection (Dozois *et al.*, 2000). Consistent with these observations, Tsh can adhere to red blood cells and also bind to extracellular matrix proteins (Kostakioti & Staphopoulos, 2004). Tsh is produced as a 140kDa protein that is processed into a 33kDa agglutinin fragment and an 106kDa fragment with mucinase activity (Kobayashi *et al.*, 2007). Interestingly, the role of autotransporter proteins in colonisation and adherence to epithelial cells has previously been suggested (Henderson *et al.*, 2004) and recently we have shown EspP, an autotransporter protein of *E. coli* O157:H7 to influence intestinal colonisation of calves (Dziva *et al.*, 2007). Intimin, an adhesin associated with selected diarrheagenic *E. coli*, was detected only in a few avian intestinal *E. coli* strains (Stordeur *et al.*, 2002). Other putative afimbrial adhesins identified by SCOTS include; TcfD and a ColV plasmid-encoded haemoglobin protease (Dozois *et al.*, 2003), but their relevance to colonisation remains to be confirmed.

**3.2. Invasive factors.** It remains unclear where and how APEC reaches the bloodstream. Avian airsacs lack resident defence mechanisms and rely on recruited inflammatory heterophils followed by macrophages (Ficken & Barnes, 1989). In vivo experiments have shown that APEC are able to survive macrophage activity (Pourbakhsh *et al.*, 1997a; Mellata *et al.*, 2003b). Whether bacteria gain entry into the bloodstream following uptake by macrophages or there is a direct invasion after damage to airsac or lung epithelia, is unknown. Entry through pulmonary lymphatics has been suggested (Cheville & Arp, 1978) and bacteria have been shown to be abundant in the blood as early as 3 hours following intra-airsac inoculation (Piercy & West, 1976; Pourbakhsh *et al.*, 1997a; Stordeur *et al.*, 2004) and aerosol inoculation (Cheville & Arp, 1978). Bacterial factors mediating translocation across the mucosal surfaces are ill-defined. In human exPEC, penetration of the blood-brain barrier has been shown to be mediated by multiple factors that include *ibeA* gene cluster (Kim, 2001). Recent work indicates that IbeA may be playing a similar role in APEC O2 strains (Germon *et al.*, 2005). In support of this, genetic surveys by PCR have established a link between the *ibeA* gene and APEC O2 strains (Germon *et al.*, 2005; Vidotto *et al.*, 2007). However, the low frequency of *ibeA* in APEC O78 strains (Germon *et al.*, 2005) and its presence in some isolates from apparently healthy birds (Rodriguez-Siek *et al.*, 2005a) implies an inconsistent requirement of this gene for invasion.

Another potential invasins identified in APEC is the homologue of the 25kDa Tia protein encoded by the *tia* locus in enterotoxigenic *E. coli* (ETEC). The *tia* locus influences adherence and invasion of cultured human ileocaecal and colonic epithelial cells by ETEC (Fleckenstein *et al.*, 1996). In APEC, the *tia* gene is located on a 56kb pathogenicity island termed PAI I<sub>APEC-O1</sub>, which also carries the *pap* operon among other putative virulence factors (Kariyawasam *et al.*, 2006a). This gene had been found to be significantly associated with strains from diseased birds (Kariyawasam *et al.*, 2006b).

Besides mediating adherence to chick explant tissues, curli have been reported to mediate invasion of eukaryotic cells (Gophna *et al.*, 2001) and invasion in the day-old chick infection model (La Ragione *et al.*, 2000b). However, the role in invasion *per se* is questionable since curli are widespread among non-pathogenic and laboratory-adapted strains.

**3.3. Iron acquisition systems.** The ability of pathogenic bacteria to sequester iron from body fluids is considered pivotal for virulence and in APEC this characteristic has been linked with lethality for day-old chicks (Dho & Lafont, 1984). Although a number of iron acquisition mechanisms exist in Gram-negative pathogens, the aerobactin system is by far the well-characterised. The system comprises genes for the synthesis of the hydroxamate siderophore aerobactin (*iuc*) and for ferric aerobactin uptake (*iut*) and these are expressed by virulent strains of *E. coli* (Warner *et al.*, 1981; Lafont *et al.*, 1987). Recently, these genes have been mapped to a conserved region of a 93-kb cluster of putative virulence genes on a ColV plasmid, pAPEC-O2-ColV (Johnson *et al.*, 2006). Transforming an avian commensal *E. coli* strain with this plasmid led to a significant increase in the strains' lethality for chicken embryos and ability to colonise the murine kidney (Skyberg *et al.*, 2006). Besides aerobactin, other iron acquisition and transport systems carried on the ColV plasmid include; salmochelin siderophore system (encoded by the *iroBCDEN* locus), *sitABC* iron transport systems and a putative iron transport system novel to APEC (*eit*) (Johnson *et al.*, 2006). The roles of these systems in virulence, independently or in combination, remain to be established. Recently, SitABCD has been shown to contribute to virulence of APEC  $\chi$ 7122 in the chicken infection model and together with MntH, mediate resistance to oxidative stress (Sabri *et al.*, 2008). Apart from plasmid-borne factors, genes encoding for ferric yersiniabactin uptake (*fyuA*) and iron-repressible protein (*irp2*) have been detected by PCR in more than 65% of strains isolated from cases of avian colibacillosis (Janssen *et al.*, 2001). Subsequent work has shown that production of yersiniabactin contributes to virulence of exPEC and *Klebsiella pneumoniae* in mice (Schubert *et al.*, 2002; Lawlor *et al.*, 2007). Additionally, *chuA* (encoding a haeme utilisation/transport protein) was detected by screening a signature-tagged mutagenesis bank of APEC O2 in chickens (Li *et al.*, 2005) suggesting a role in virulence.

**3.4. Serum resistance mechanisms .** Resistance to complement-mediated lysis and opsonophagocytosis is believed to play an important role in APEC virulence (Vidotto *et al.*, 1990; Nolan *et al.*, 1992a; 1993). A region on ColV plasmids harbouring *traT* and *iss* was first linked to serum resistance and virulence (Binns *et al.*, 1979). These genes encode for outer membrane proteins and their role in serum resistance was confirmed by insertional mutagenesis (Sukupolvi *et al.*, 1987; Wooley *et al.*, 1993). TraT and Iss are believed to prevent deposition of the membrane attack complex (MAC) of complement by an unknown mechanism. TraT is thought to antagonize C3

deposition (Aguero *et al.*, 1984) and to inhibit formation of the C5b6 complex (Pramoonjago *et al.*, 1992). Whilst TraT could be linked to serum resistance in APEC (Pfaff-McDonough *et al.*, 2000), this could not be established in an isolated study with bovine *E. coli* isolates from cases of mastitis (Nemeth *et al.*, 1991). The *iss* gene occurs more frequently in APEC than strains from apparently healthy birds (Pfaff-McDonough *et al.*, 2000; McPeake *et al.*, 2005; Rodriguez-Siek *et al.*, 2005a). Compared to LPS and K1 capsule, *Iss* was reported to play a subtle role in resistance of APEC to serum (Mellata *et al.*, 2003a) but it is required for full virulence (Tivendale *et al.*, 2004). It has already been suggested that there could be a compelling but imperfect relationship between complement resistance, virulence and the presence of *iss* (Nolan *et al.*, 1992a) and that high resistance to complement may be necessary but not sufficient for virulence (Chaffer *et al.*, 1999).

Besides TraT and *Iss*, a 16.2kDa protein believed to mediate complement resistance was identified by screening a random transposon mutant library of APEC (Nolan *et al.*, 1992b).

**3.5. Antiphagocytic activity.** Certain O and K antigens on the bacterial surface are well-established to prevent opsonisation and phagocytosis. The K1 antigen contains poorly immunogenic N-acetylneuraminic acid and has been reported to prevent activation of the alternate complement pathway by NMEC (Pluschke *et al.*, 1983), despite a contradicting report (Wooley *et al.*, 1993) which found no direct role of K1 capsule and TraT in mediating complement resistance of APEC. TraT protein acts as an inhibitor of phagocytosis by impeding C3 deposition (Aguero *et al.*, 1984). Seven of the 31 attenuated APEC-O2 mutants contained transposon insertions in genes encoding for capsule and LPS, indicating crucial roles of these bacterial factors in virulence (Li *et al.*, 2005) though direct mediation of antiphagocytic activity was not established. Apart from mediating iron and manganese transport, *sitABCD* operon has also been suggested to confer resistance to oxidative stress (Sabri *et al.*, 2006) possibly required during interaction with phagocytes.

**3.6. Toxins.** A vacuolating autotransporter toxin, demonstrable in cell-free culture supernatants (Salvadori *et al.*, 2001) and encoded on a pathogenicity island termed VAT-PAI, contributes to the virulence of APEC (Parreira & Glyes, 2003). Other forms of toxins reported in APEC strains, but with obscure roles in pathogenesis include; enterohaemolysin, cytotoxic necrotising factor 1, cytolethal distending toxin (Blanco *et al.*, 1997) and a cytotoxin designated VT2y (Parreira & Yano, 1998). Shiga toxin gene sequences have been detected in avian *E. coli* by PCR (Parreira & Gyles, 2002), but evidence of their expression is limited. Blanco *et al.* (1997) observed a cytotoxic response on HeLa but not Vero cells in 7% of the strains indicating the presence of a toxin not related to Shiga toxins. Recently, a potent mediator for apoptosis (caspase 3/7-induced) and cytotoxic activity was reported following a 6-hour infection assay employing an immortalised macrophage cell line by an APEC strain (Bastiani *et al.*, 2005) but factors mediating those processes were not identified. Other toxins reported in APEC strains include; the chicken-lethal toxin (Truscott, 1973), the heat-labile enterotoxin (Tsuji *et al.*, 1990) and a homologue of the heat-stable enterotoxin 1 (AstA) of enteroaggregative *E. coli* (Janssen *et al.*, 2001). It is unclear whether these homologues mediate APEC virulence in a similar manner to their counterparts. The impact of toxoid-based vaccines or of passive immunisation with anti-toxin antibodies on APEC pathogenesis has received little study.

**3.7. Virulence gene regulators.** It well-known that expression of bacterial virulence is regulated by conserved global systems that sense and adapt to changes in the environment. One such well-recognised system is the BarA-UvrY two component, which has been shown to regulate APEC virulence by; i) down-regulating type 1 and Pap fimbriae, ii) increasing susceptibility to oxidative stress and iii) reducing the amount of surface polysaccharide (Herren *et al.*, 2006). In addition, a specific phosphate transport system (Pts) has also been linked with virulence in APEC  $\chi$ 7122 strain (Lamarche *et al.*, 2005). The Pts system mediates uptake of phosphate by serving as a primary sensor for extracellular phosphate levels and its inactivation results in constitutive expression of the Pho regulon. Inactivation of the *pts* system has been reported to render an exPEC strain avirulent (Daigle *et al.*, 1995). In APEC, PhoB was identified to be a putative virulence factor by SCOTS (Dozois *et al.*, 2003) and subsequent *in vivo* experiments with an isogenic *pts* mutant confirmed the findings (Lamarche *et al.*, 2005). Other regulatory networks in APEC remain to be identified.

**3.8. Unclassified factors.** Subtractive hybridisation studies with an APEC O78 strain first revealed a gene of the degenerate Type III secretion system (ETT2) (Mokady *et al.*, 2005). Subsequent work established the presence of an ETT2 cluster among APEC strains that underwent mutational attrition but remained essential for APEC virulence (Ideses *et al.*, 2005b). In *E. coli* O157:H7, the cryptic ETT2 island does not function as a protein secretion system but as a regulator of other virulence determinants (Zhang *et al.*, 2004). Recently, a *selC*-associated genomic island containing putative mobile genetic elements and genes encoding for proteins associated with carbohydrate assimilation has been identified (Chouikha *et al.*, 2006). Mutation of the region associated with carbohydrate assimilation was shown to reduce APEC virulence in a chicken infection model. It is unclear whether this region directly affects virulence since the mutant exhibited a lower *in vitro* growth rate than the parent strain.

A number of other putative virulence factors were identified by SCOTS (Dozois *et al.*, 2003) and GSSH (Stocki *et al.*, 2002; Schouler *et al.*, 2004; Kariyawasam *et al.*, 2006b; 2007) including genes of unknown function, but their roles in pathogenesis remain to be validated.

**4. Prospects for the identification of novel APEC virulence determinants.** Though GSSH and SCOTS have identified APEC-specific and *in vivo*-induced genes respectively, approaches that define the function of such genes in a natural host like signature-tagged transposon mutagenesis (STM) and targeted mutagenesis are also required to understand APEC virulence. STM has been used widely to identify bacterial genes required for colonisation and survival within the host (reviewed in West *et al.*, 2003). Using this approach, we identified novel intestinal colonisation factors of enterohaemorrhagic *E. coli* (EHEC) serotypes O157:H7 and O26:H- in cattle (Dziva *et al.*, 2004; van Diemen *et al.*, 2005), factors mediating carriage and systemic virulence of *Salmonella enterica* in cattle, pigs and poultry (Bispham *et al.*, 2001; Morgan *et al.*, 2004; Carnell *et al.*, 2007). STM has also been successfully used to identify genes required for systemic virulence and intestinal carriage of NMEC (Badger *et al.*, 2000; Martindale *et al.*, 2000) and UPEC involved in an ascending urinary tract infection model (Bahrani-Mougeot *et al.*, 2002). Recently, this technique has enabled the identification of APEC O2 genes required for septicaemia in the chicken following intra-tracheal inoculation (Li *et al.*, 2005). A pool of uniquely-

tagged random transposon mutants is simultaneously inoculated into the host and after a suitable time, a representative output pool is recovered. The unique tags are then amplified from the input and output pools and hybridised to a template on which each of the mutants in the input pool is represented (Fig. 1). By comparing the composition of input and output pools, mutants unable to survive in the host can be identified and the disrupted genes subsequently identified by subcloning and sequencing of the site of transposon insertion. This offers the advantage that almost a hundred individually-tagged random mutants can be screened simultaneously for defects in virulence. Further STM studies with different APEC serogroups in different avian hosts may identify conserved factors that play common roles in disease pathogenesis.

Approaches to identify bacterial factors that elicit protective immunity also hold promise. The value of antibodies in homologous protection against APEC infection has been reported by several workers. Chickens with greater IgG, IgA and IgM responses to selected cell surface antigens of APEC were protected against experimental challenge than their unvaccinated counterparts (Kariyawasam *et al.*, 2002). Correlation between antibody titre and lesions was observed following vaccination of chickens with an ultrasonic inactivation-based *E. coli* vaccine (Melamed *et al.*, 1991). Even passively transferred antibodies protected against homologous challenge with respective virulent APEC strains (Arp, 1980; Bolin & Jensen, 1987; Kariyawasam *et al.*, 2004a). Furthermore, rapid clearance of an APEC strain from the bloodstream of turkeys was shown to be mediated by antibody-dependent phagocytosis (Arp, 1982). Identification of factors that evoke significant humoral responses during the infection and convalescence by genomic and proteomic strategies therefore offers a rational platform for the design of protective vaccines. One such strategy is the *in vivo*-induced antigen technology (IVIAT) approach (reviewed in Rollins *et al.*, 2005). IVIAT involves cloning of fragments of genomic DNA from the pathogen in an inducible expression vector to produce an expression library that is probed with convalescent serum pre-absorbed with *in vitro*-grown bacteria (Fig. 2). Dominant immuno-reactive clones are identified, mapped to the APEC genome and these can further be validated *in vivo*. However, the high likelihood of pre-absorbing antibodies directed against surface antigens expressed *in vitro* but playing important roles *in vivo* presents a severe limitation to the IVIAT approach. An alternative approach is to resolve bacterial proteins by 2-dimensional gel electrophoresis and transfer them to a membrane that is then probed with host antisera to identify immunogenic constituents by mass spectrometry. This immuno-proteomic approach has successfully led to the identification of important antigens in other bacterial pathogens; *Klebsiella pneumoniae* (Kurupati *et al.*, 2006) and uropathogenic *E. coli* (Hagan & Mobley, 2007). Surface-localised antigens are likely to play an important role in pathogenesis and also stimulate protective immunity hence the process can be focussed on sheared or outer membrane components from the cell surface. This strategy has led to the identification of 110 surface proteins of *Campylobacter jejuni*, eight of which were further evaluated for protective efficacy in the mouse infection model (Prokhorova *et al.*, 2006). In cases where convalescent sera cannot be obtained, surface-exposed proteins can be identified using a similar proteomic approach following labelling of bacterial cell surface with biotin followed by detection of labelled proteins using streptavidin conjugated to a fluorochrome or enzyme.

Although the authors have selected to highlight STM and IVIAT, it should be emphasised there are other alternative genome-based approaches equally capable of defining APEC activity *in vivo*. These include *in vivo* expression technology (IVET;

reviewed in Rediers *et al.*, 2005), transposon-site hybridisation (TRASH; Chan *et al.*, 2005) and reporter gene fusions to detect bacterial gene expression *in vivo* (Chalfie *et al.*, 1994). The availability of the APEC O1 genome sequence (Johnson *et al.*, 2007a) also allows automatic *in silico* prediction of novel virulence loci which would require validation for their roles in pathogenesis. The advent of high-throughput pyrosequencing methods promises the availability of many more APEC genomes in years to come.

**5. Concluding remark.** Colibacillosis may be controlled by antimicrobial agents, but the encounter of residues of such agents in food is of major concern (Singer and Hofacre, 2006). Control by non-antibiotic interventions therefore presents an alternative rational approach. Utilisation of bacteriophages for the control of APEC has been explored but this remained unpopular (Huff *et al.*, 2003; 2005). Several other alternatives for interventions to reduce carriage of pathogens in food-producing animals have been discussed elsewhere (Doyle & Erickson, 2006) and it is clear that vaccination provides the most rational option. In previous studies, vaccination with live attenuated mutants generated serotype- and strain-specific protection (Kwaga *et al.*, 1994; Peighambari *et al.*, 2002; Kariyawasam *et al.*, 2004b), hence the need to develop cross-protective vaccines. The search for such vaccines should remain at the forefront of future APEC research and by combining bioinformatics, genomic and proteomic approaches it can be expected that more vaccine candidate will be identified. Promisingly, these approaches are already proving successful in the identification of novel vaccine candidates in other pathogenic bacteria. Recent comparative genomic analysis led to the identification of candidates for subunit vaccines against exPEC (Durant *et al.*, 2007). And following the successful application of reverse vaccinology to identify potential vaccine candidates for *Neisseria meningitidis* (Pizza *et al.*, 2000), multiple genomic screening enabled the identification of a universal Group B *Streptococcus* vaccine candidate (Maione *et al.*, 2005). Furthermore, software for *in silico* identification of best vaccine candidates from the whole proteomes of bacterial pathogens has been developed (Vivona *et al.*, 2006). Sequencing of the genomes of more APEC strains representing all predominant pathogenic serotypes will aid the reverse vaccinology approach. However, it should be remembered the predominant APEC serotypes O1, O2 and O78 potentially employ different mechanisms at different stages of infection (Mokady *et al.*, 2005; Ron, 2006), which could pose a barrier to the design of cross-protective vaccines.

As for other pathogens, a key challenge in the post-genomic era will be to define the role of the encoded APEC genes in pathogenesis and transmission and to identify cellular constituents able to elicit protective immunity. For such a heterogenous group of pathogens, it will be important to define the impact of variation in the repertoire, sequence and expression of encoded gene if we are to derive broadly cross-protective intervention strategies in the future.

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**Figure legends**

**Figure 1.** *Principle of signature-tagged transposon mutagenesis.*

**Figure 2.** *In vivo-induced antigen technology (IVIAT).*

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Figure 1

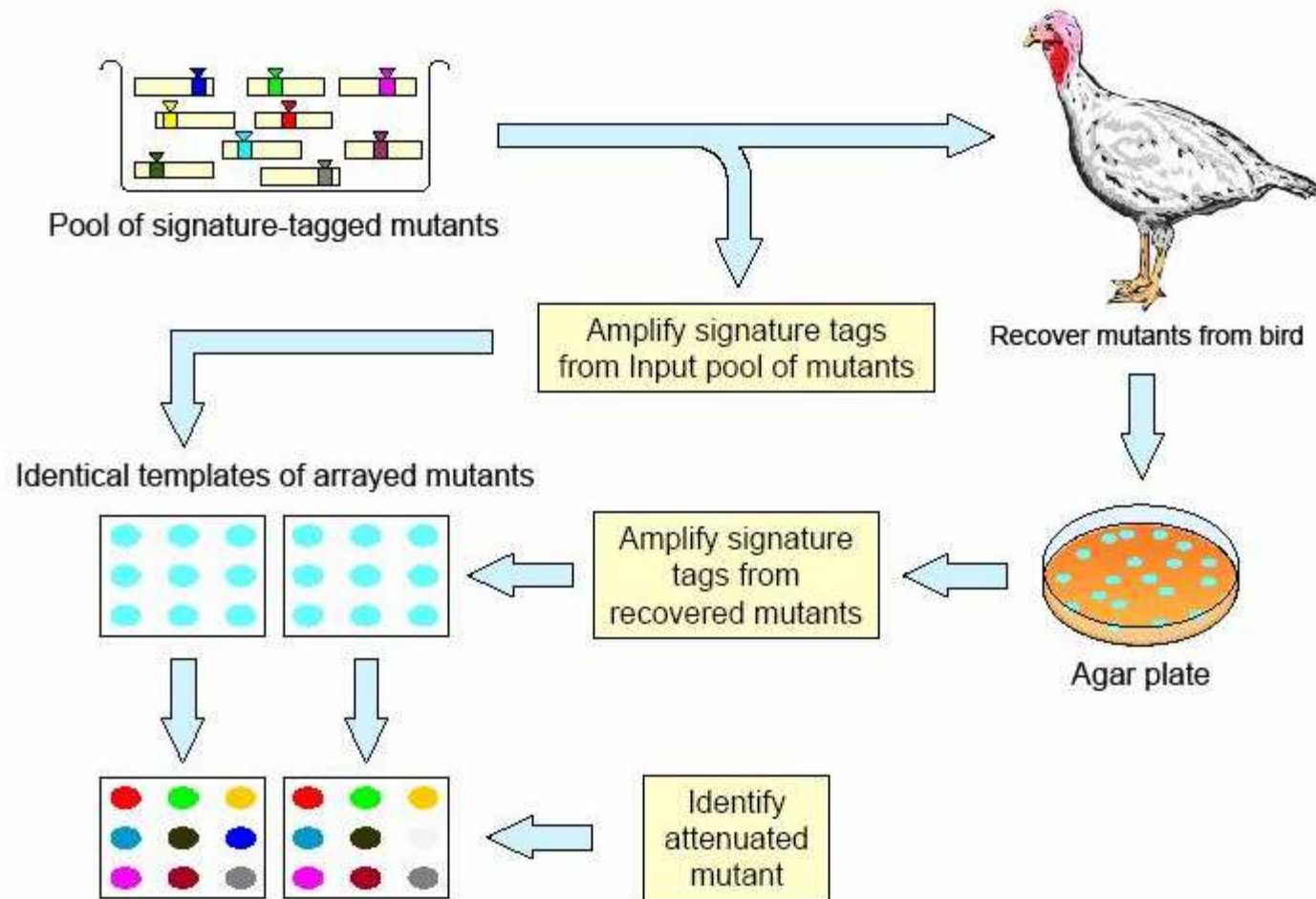
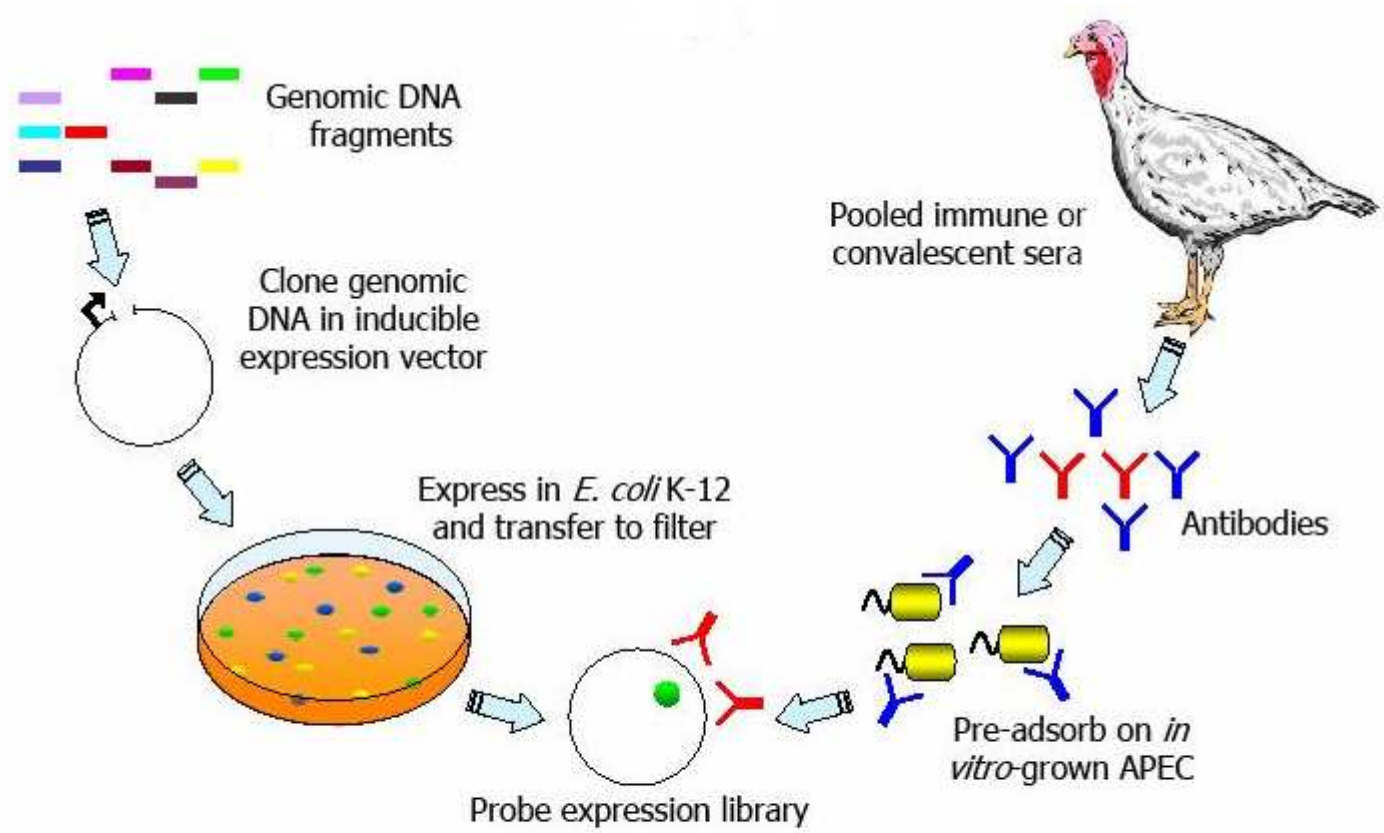


Figure 2



- Screen sera from APEC-infected turkeys and chickens against expression libraries
- Identify immuno-reactive clones
- Validate findings and assess vaccine potential