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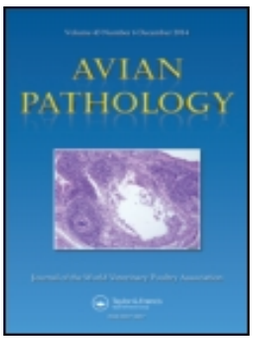

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The bird's immune response to avian pathogenic *Escherichia coli*

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Abstract

Avian pathogenic *Escherichia coli* (APEC) cause colibacillosis in birds, a syndrome of severe respiratory and systemic disease that constitutes a major threat due to early mortality, condemnation of carcasses and reduced productivity. APEC can infect different types of birds in all commercial settings, and birds of all ages although disease tends to be more severe in younger birds likely a consequence of an immature immune system. APEC can act as both primary and secondary pathogens, with predisposing factors for secondary infections including poor housing conditions, respiratory viral and *Mycoplasma spp.* infections or vaccinations. Controlled studies with APEC as a primary pathogen have been used to study the bird's immune response to APEC, although it may not always be representative of natural infections which may be more complex due to the presence of secondary agents, stress and environmental factors. Under controlled experimental conditions, a strong early innate immune response is induced which includes host defence peptides in mucus and

a cellular response driven by heterophils and macrophages. Both antibody and T-cell mediated adaptive responses have been demonstrated after vaccination. In this review we will discuss the bird's immune response to APEC as primary pathogen with a bias towards the innate immune response as mechanistic adaptive studies clearly form a much more limited body of work despite numerous vaccine trials.

Keywords

APEC, *E. coli*, avian, chicken, innate, adaptive, immunity, vaccine

Non-pathogenic *E. coli* are a normal inhabitant of the bird's gastrointestinal tract and to a lesser extent the pharynx and trachea, and generally do not induce disease. However, some variants are adapted to cause disease in birds and are broadly grouped in the pathotype of avian pathogenic *E. coli* (APEC). APEC infections are grouped under the term colibacillosis but can present in diverse ways, including severe respiratory infections (e.g. airsacculitis), fibrinous deposits around visceral organs in which the bacteria reach high numbers (e.g. perihepatitis and pericarditis), reproductive tract infections (salpingitis), naval infections in neonates (omphalitis), and rapidly fatal septicaemia. Infections can involve combinations of these presentations owing to the ability of APEC to translocate from mucosal surfaces to systemic sites. APEC constitute a very diverse group of pathogens with serogroups O1, O2 and O78 thought to be amongst the most prevalent globally and often used in controlled studies (reviewed in Guabiraba & Schouler, 2015).

Main APEC pathogenic factors

APEC serotypes are characterised by their O, K and H antigens. It is thought that the immune response in poultry is primarily directed against the O antigen (Pluschke *et al.*, 1983). The O (somatic) antigen refers to the antigenic portion of lipopolysaccharide (LPS), a component of the

APEC cell wall that acts as an important pathogen-associated molecular pattern (PAMP). LPS binds to LPS binding protein (LBP) which in turn initiates innate immune signalling via receptors such as CD14 or toll-like receptor 4 (TLR) on innate immune cells. K (capsular) antigens refer to polysaccharides on the surface of the bacteria and are associated with pathogenicity as they are thought to contribute towards the evasion of phagocytosis by the cellular innate immune response and resistance to complement-mediated killing and opsonisation (Mellata *et al.*, 2003). K antigens also often mimic carbohydrate moieties found in the host, making the bacteria more difficult to recognise as non-self. The H (flagellar) antigen is the major component of the flagella. As such the H antigens are directly linked to the motility of APEC, which is known to be required for virulence. Targeted and genome-wide mutagenesis has identified other virulence factors across a range of APEC, including fimbriae, non-fimbrial adhesins and invasins, the TraT and Iss serum-resistance determinants, a temperature-sensitive haemagglutinin and iron acquisition systems (reviewed in Dziva & Stevens, 2008). Significant variation can exist in the repertoire of virulence-associated genes across APEC, as first revealed by genome sequencing of APEC O1 and O78 strains that varied by over a thousand chromosomal genes and four plasmids, including in the complement of known virulence factors (Dziva *et al.*, 2013). This has been reinforced by wider genome sequencing studies (Huja *et al.*, 2015). As such, caution is required when interpreting host responses to APEC, as they may be influenced by properties of the bacteria and not apply universally to the pathotype.

Soluble immune mediators

APEC enter the avian host often through mucosal colonisation at the site of entry such as the respiratory tract. As such the bird's first defence mechanisms are related to mucosal surfaces including physical, chemical and cellular barriers. Host defence peptides (HDPs) are small cationic peptides with important functions in antimicrobial defence but also in the regulation of innate immune responses, including in the context of *E. coli* (Xiao *et al.*, 2006). In chickens, two major

classes of HDP have been identified, cathelicidins (CATH) and defensins originally called gallinacins (reviewed in Cuperus *et al.*, 2013). CATH-1, -2 and -3 and CATH-B1 were described as well as avian β-defensins (AvBD) 1–14 thus far in chickens. Cathelicidins are an important component of heterophilic granules, whereas defensins are more diversely expressed. HDPs release can result in direct killing of pathogens, alternatively after phagocytosis the fusion of cytoplasmic granules containing HDPs with the phagosome of heterophils can lead to killing of the invading pathogen (reviewed in Cuperus *et al.*, 2013).

Several studies investigated the effect of chicken cathelicidins in the context of *E. coli* infections in mice, with view of their use as an alternative to antibiotics. These studies showed that chicken cathelicidins possess potent antimicrobial activity against a range of bacteria including various multiresistant bacterial families including *E. coli* 38.34 without inducing strong resistance (Veldhuizen *et al.*, 2013). Cathelicidins also possess immunomodulatory and protective properties mediated through the recruitment of neutrophils and activation of macrophages by inducing the expression of inflammatory mediators such as IL-1β, CCL2, and CCL3 in mice and murine RAW264.7 macrophages (Bommineni *et al.*, 2014). This demonstrates the antimicrobial and immunomodulatory activity of chicken cathelicidins likely to be important in the fight against APEC in birds.

The expression and immunomodulatory function of cathelicidins and defensins was described in a number of *in vitro* and *in vivo* studies in chickens and ducks, with particular focus on macrophages. Chicken CATH-2 strongly enhances DNA-induced activation of chicken macrophages because of enhanced endocytosis of DNA–CATH-2 complexes (Coorens *et al.*, 2015). After endocytosis, *E. coli* DNA was liberated from the complex because of proteolytic breakdown of CATH-2, after which chicken TLR21 was activated. This led to increased cytokine mRNA expression including IL-1β, IL-6, IL-10 and CXCLi2, and NO production of the chicken macrophage cell line HD11. CATH-2 kills *E. coli* O6 and APEC O78 in an immunogenically silent fashion by permeabilising the bacterial inner membrane

(Coorens *et al.*, 2017). Subsequently CATH-2 binds the outer membrane-derived lipoproteins and LPS to inhibit TLR2 and TLR4 activation in J774.A1 murine macrophages and chicken peripheral blood mononuclear cells (PBMC), thereby inhibiting macrophage activation and a potentially harmful immune response. *In ovo* pre-treatment of broilers with the D analog of chicken cathelicidin-2 (D-CATH-2) resulted in partial protection of hatchlings from a relatively high challenge dose of 10^6 CFU APEC O78 (Cuperus *et al.*, 2016). Using the HD11 cell line and primary monocyte-derived macrophages it was also shown that CATH-B1 has immunomodulatory functions as treatment of these macrophages resulted in downregulation of pro-inflammatory cytokines such as IL-6 and IL-1β and enhanced phagocytosis post APEC O78 strain 506 (Peng *et al.*, 2020). However, the antibacterial activity was lower than that of CATH-1, CATH-2 and CATH-3.

The antimicrobial activity of chicken β-defensin 4 and 10 was demonstrated *in vitro* in minimum inhibitory concentration (MIC) assays in culture broth using various bacterial families including an *E. coli* O6 strain (Yacoub *et al.*, 2015). This study revealed a stronger antibacterial effect of chicken β-defensin 10 against all bacterial families tested whilst β-defensin 4 also possessed antibacterial activity. In ducks, up-regulation of β-defensin 2 post high dose APEC O1 infection was also shown (Li *et al.*, 2016).

The serum complement system is an important antimicrobial defence mechanism once mucosal barriers are breached. Only a couple of minutes are required between detection of invading bacteria and subsequent neutralisation via direct permeabilization of their membrane by complement components or indirect killing via recognition by innate immune cells of opsonised bacteria. Activation of the lectin complement pathway can occur when mannose binding lectin (MBL) has bound to a microorganism. It promotes killing of the microorganism either by acting directly as an opsonin or via the MBL-associated serine proteases (MASP-1 and MASP-2) making MBL an important part of the innate immune defence. Chickens with higher levels of MBL in serum are less prone to disease (Norup *et al.*, 2009). While complement can play an important protective role, many APEC

have adapted to survive in serum, with O and K antigens and surface-associated proteins such as Iss/Bor playing key roles in resistance.

Acute phase proteins (APPs) have been described in chickens in the context of APEC infections. As in mammals, inflammation in chickens is thought to be accompanied by a number of systemic and metabolic changes termed the acute phase response, with differing kinetics ranging from some APPs showing high expression with a couple of hours and others reaching their peak a couple of days post-infection. In chickens, APPs are thought to be mainly produced in the liver, but expression of the long pentraxin PTX3 gene was recently shown in a variety of other tissues such as the skin, kidney, spleen, lung and trachea also (Burkhardt *et al.*, 2019). PTX3 was shown to be a major acute phase protein and possibly a marker to monitor inflammatory conditions in poultry flocks. Strong up-regulation of PTX3 after APEC O1 infection correlated with the bacterial counts in the spleen of chickens (Burkhardt *et al.*, 2019). After APEC 02, 078 or 0111 infection, ceruloplasmin content and fibrinogen levels also increased significantly post-infection (Butler *et al.*, 1972; Piercy, 1979; Georgieva *et al.*, 2010).

Altogether, these studies indicate an important role for soluble mediators at the mucosal surfaces and in the circulation which may directly kill invading APEC, facilitate APEC recognition and killing by innate immune cells or contribute to the modulation of the innate immune response. Further studies are required to define the activity of the avian soluble mediators described to date against APEC of varying types.

The epithelial barrier

Epithelial barriers are thought to play important roles in the innate immune response to APEC and to limit APEC dissemination, but little is known in part due to the lack of good *ex vivo* models. From mammalian studies it is known that intestinal epithelial cells form a highly organised cellular system

which not only forms an important physical barrier but also plays important roles in maintaining homeostasis and in the local innate immune response (reviewed in Peterson & Artis, 2014). When invading the host, bacteria first encounter mucus and soluble factors at these epithelial surfaces before they adhere and invade. In the context of mammalian *E. coli* infection in the intestinal tract, it was shown that tight junctions and adherens junctions are critical elements of the epithelial barrier and that *E. coli* may also selectively target protein components of these junctions, leading to increased barrier permeability (reviewed in Shawki & McCole, 2017). In chickens, primary intestinal epithelial cells were able to take up and process pHrodo™ green *E. coli* BioParticles®. The immune response of these cells to *E. coli* derived LPS and LTA included increased expression of IL-6 and IL-18 mRNA as well as the expression of the AAP avidin and lysozyme (Shira & Friedman, 2018). In the chicken lung, it was macroscopically shown that APEC associate with epithelial cells early after infection but the immune response was not studied (Pourbakhsh *et al.*, 1997a; Pourbakhsh *et al.*, 1997b; Swamy *et al.*, 2001; Edelman *et al.*, 2003). Inflammatory reactions and recruitment of innate immune cells not only occur in the lung of chickens, but also in the trachea and air sacs after APEC O78 strain 506 infection (Dwars *et al.*, 2009). How exactly APEC enter the avian host and subsequently disseminate to organs is still poorly understood. It is thought that APEC may disseminate transcellularly through non-phagocytic cells such as the tracheal epithelium or fibroblasts, and both air sacs and lungs may be the portal of entry for APEC into the systemic circulation, but avian factors involved in cell penetration and APEC systemic dissemination may vary between APEC serotypes and still need to be fully elucidated (Pourbakhsh *et al.*, 1997a; Pourbakhsh *et al.*, 1997b; Pourbakhsh *et al.*, 1997c).

The tracheal epithelium is covered by a layer of mucus and ciliary movements enable clearance of mucus and particles from the airway. It was shown that APEC strain YA21 adheres to and invades primary chicken tracheal epithelial cells, although invasion rate was very low with only 0.3% of the 10^8 CFU APEC inoculum found inside these cells (Ramírez *et al.*, 2009). In comparison, even less adhesion and no invasion were observed after inoculation with the non-pathogenic *E. coli* strain

DH5α. *In vivo* intra-tracheal inoculation of week-old chicks with $5 \cdot 10^6$ CFU APEC strain AE17 resulted in oedema, heterophil infiltration and hyperaemia appeared as early as 8 h post-infection (Song *et al.*, 2020). RNA sequencing showed many genes were dynamically expressed after infection with cytokine–cytokine receptor interaction and toll-like receptor signalling enriched at 4, 8 and 12 hpi. Amongst the up-regulated genes were *IL1B*, *IL8*, *IL8L1*, *CCL19* and *CCL20* (Song *et al.*, 2020). It is noteworthy that many *of* the pre-disposing factors for secondary APEC infection are associated with damage to epithelia, and in the case of infectious bronchitis virus, impaired mucociliary clearance from the lungs.

It was shown *in vitro* that embryonic primary type II pneumocytes belonging to the squamous epithelial cells possess important roles as an entry barrier for pathogens including APEC and as modulators of the local immune response. The transcriptome of primary chicken type II pneumocytes after stimulation with a high dose of APEC O78 (a multiplicity of infection (MOI) of 100) suggested that the major enriched pathways were related to the NF-κB signalling pathway, apoptosis, tight junctions, and cytokine-cytokine receptor interactions, with *IL1B*, *IL6* and *IL18* up-regulated (Peng *et al.*, 2019b). Several plant-derived substances can modulate the type II pneumocyte immune response to APEC, for example rutin, astragaloside IV, α-cyperone, baicalin, and schizandrin may reduce cell damage of type II cells after APEC O78 exposure (Zhang *et al.*, 2014; Zhang *et al.*, 2016; Peng *et al.*, 2018b; Peng *et al.*, 2019a; Peng *et al.*, 2019c; Yuan *et al.*, 2020a; Yuan *et al.*, 2020b). However, further studies are required to study the detailed protective functions of these plant-derived compounds.

A model of embryonic primary chicken aortic endothelial cells (pchAEC) was shown to be susceptible to infections with various avian pathogens including APEC (Lion *et al.*, 2019). APEC O2 strain BEN2908 showed similar capacity to adhere to pchAEC as compared to the chicken lung epithelial cell line CLEC213 and the chicken hepatocellular carcinoma cell line LMH, but the invasion rate was markedly lower in pchAEC especially compared to the LMH cells. APEC O78 strain 506 (MOI 25) was

able to adhere and subsequently invade the CLEC213 cells and induced an increase in IL-8 mRNA expression, a chemo-attractant of macrophages and heterophils (Mol *et al.*, 2019). APEC BEN2908 (MOI 10) can manipulate cellular endocytic pathways to invade the human type II pneumocyte cell line A549 and the LMH cells where it is able to survive for several days (Chanteloup *et al.*, 2011).

Matter and colleagues described the role of chicken fibroblasts, using the chicken non-phagocytic fibroblast cell line CEC-32, in the immune response to APEC, adherence, invasion, cytotoxicity and induction of caspase-3/7. They used eight APEC strains with differing pathogenicity in an attempt to understand the mechanisms by which the pathogens gain access to the host bloodstream (Matter *et al.*, 2011). Independent of their pathogenicity, all bacterial strains showed similar levels of adhesion, lactate dehydrogenase release as measure of cytotoxicity and caspase-3/7 activation. However, APEC strain MT78 showed high invasion capability comparable to the highly invasive *Salmonella* Typhimurium strain SL1344 used for comparison. Thus, fibroblasts adjacent to the epithelia may also play roles in APEC dissemination.

Altogether, these studies indicate that epithelia play an important role in the defence against APEC by providing a physical barrier and contributing to the modulation of the local innate immune response. However, current APEC-specific literature especially of epithelia in the respiratory tract often the site of entry for APEC is very limited, at least in part due to the lack of good *ex vivo* models. In experimental models, doses required to elicit systemic disease can vary markedly between the oral and respiratory routes, and the extent to which this is due to events at the epithelial layer, or distinct gene expression or microbiota is ill-defined.

Cellular innate immune response

Heterophils (avian equivalent of neutrophils), macrophages, dendritic cells and thrombocytes are thought to be important in the fight against APEC. The strategic location of macrophages and

dendritic cells in the mucosa of larger airways and air sacs of chickens was studied in detail (Dwars *et al.*, 2009; Sutton *et al.*, 2018). Heterophils can be quickly recruited to the site of infection and heterophils and macrophages identified macroscopically located in the spleen and liver were shown to phagocytose bacteria which gained access to the circulation (Arp, 1982; Arp & Cheville, 1981). Complement components and antibodies to surface proteins of APEC can act as opsonins and promote phagocytosis and killing of bacteria (Arp, 1982; Arp, 1985; Arp & Cheville, 1981). Immunomodulation of innate immune cells by avian host defence peptides was also shown and is described above. Chicken thrombocytes were found to phagocytose bacteria but are less phagocytic than heterophils, however killed bacteria were not included in parallel to control for bacterial invasion (Wigley *et al.*, 1999). In the latter study, oxidative burst activity was generated upon activation of thrombocytes with various bacterial species including APEC O2 strain F77 and this indicates that thrombocytes may play a role in innate immunity to APEC in chickens.

Innate immune cells recognise distinct PAMPs on the bacterial surface which in turn activates intracellular signalling pathways resulting in antimicrobial activities and the production of pro- and anti-inflammatory cytokines. Amongst these Toll Like Receptors TLR2, TLR4, TLR5 and TLR21 were shown to be involved in bacterial sensing in birds (reviewed in Keestra *et al.*, 2013). TLR2 recognises peptidoglycan, TLR4 binds to LPS, TLR5 recognises flagellin and TLR21 (not found in mammals) unmethylated CpG DNA commonly found in bacteria. In the context of APEC, little is known about mechanistic details of these signalling cascades, which could potentially differ between serotypes and strains owing to ligand variation. It was shown in transcriptomic studies that TLRs are upregulated after APEC infection in different tissues of chickens such as the lung and spleen, but functional data are lacking (Sandford *et al.*, 2011; Nie *et al.*, 2012; Sandford *et al.*, 2012a; Sandford *et al.*, 2012b; Sun *et al.*, 2016). Studies in ducks indicated that beside TLRs, other intracellular sensors such as the nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 (NLRP3) and the class II major histocompatibility complex trans-activator (CIITA), play important

roles in the regulation of the innate immune responses to APEC (Li *et al.*, 2016; Li, *et al.*, 2017; Li *et al.*, 2018).

Pourbakhsh and colleagues studied systemic translocation of strains of varying virulence following delivery into the respiratory tract. They inoculated 12-day old broiler chickens via the air sac with 10^8 CFU of either highly pathogenic strains (TK3, CN33 and CN151; O1, O78 and O2 respectively) or less pathogenic strains (CN139, CN165 and MT78; O78, O78 and O2 respectively) and discovered colonisation of the respiratory tract and the liver, spleen and kidneys within 6 hpi (Pourbakhsh *et al.*, 1997a). However, higher loads of circulating APEC in blood were detected for the more pathogenic APEC strains. They further showed that the most pathogenic strains are more resistant to intracellular killing by blood-derived macrophages and heterophils and show higher survival rates in serum bactericidal assays. Other studies using high dose of APEC O1 and O78 demonstrated rapid colonisation of the respiratory tract and organs with significant bacterial loads detectable as early as 3 hpi, reaching peak bacterial loads around 6-12 hpi (DeRosa *et al.*, 1992; Pourbakhsh *et al.*, 1997b; La Ragione *et al.*, 2000). Overall, in these studies colonisation was accompanied by a significant early heterophil and macrophage influx into lungs and air sacs based on microscopical analysis (DeRosa *et al.*, 1992; Pourbakhsh *et al.*, 1997a; Pourbakhsh *et al.*, 1997b). A detailed histological study of 5-week old layer chickens intratracheally inoculated with 10^9 APEC (pathogenic MT78 (O2), IMT5155 (O2), UEL17, and non-pathogenic *E. coli* IMT5104 (O8)), demonstrated differential dissemination of pathogenic and non-pathogenic strains but interestingly a similar amount of lung inflammation post-infection with all four strains (Horn *et al.*, 2012). Significant localised inflammation in the lung and influx of phagocytic cells was observed from 12 hpi, with heterophils and macrophages undergoing apoptosis in the inflamed regions. Significant increases in macrophage numbers in blood and splenocytes were also shown after intratracheal inoculation of commercial broilers with APEC 506 (O78) up to seven days post-infection (Ariaans *et al.*, 2008).

In a more recent study, a strong heterophil and macrophage influx into the lungs of 6-week old *CSF1R*-reporter transgenic layer chickens was observed after intra-air sac infection with either APEC O1 or APEC O2 expressing green fluorescent protein (Alber *et al.*, 2019). For the first time this study unambiguously identified these infected cells as heterophils and macrophages by a panel of surface markers using flow cytometry and in-depth transcriptomic analysis. Transcriptomic analysis revealed a less important role for dendritic cells, as marker genes for these cells decreased within 6 hpi suggesting that the cellular influx was dominated by macrophages. Interestingly however, this study revealed that only a small fraction of macrophages was APEC positive whereas the majority of the bacteria were detected in heterophils. Further differences in the immune responses to APEC O1 or APEC O2 were shown with immune pathways repressed to a greater extent or less activated in birds inoculated with APEC O2 compared to APEC O1.

Several studies investigated the immune response of macrophages after APEC inoculation *in vitro*, with a particular focus and the phagocytic and bactericidal activity and immunomodulatory responses. It was shown that peritoneal macrophages isolated from 4-week old broilers possess potent microbicidal activity when infected with a relatively low MOI of 0.5 of *E. coli* E/2/64 (Harmon & Glisson, 1989). Gene expression profiles of chicken monocyte-derived macrophages (MDM) and HD11 cells were analysed up to 24 hpi with APEC V-G (MOI 100). This study showed significant induction of the chemokines *CCL5*, *CCLi7* and *CXCLi1*, *iNOS* and peroxiredoxin 1 associated with oxidative burst, and avidin post APEC infection (Lavric *et al.*, 2008). It also showed a clear difference in the response of HD11 cells between APEC or *M. synoviae* inoculation demonstrating that these cells efficiently recognise different pathogens, a difference which may also hold true for different APEC serotypes.

A number of other studies used the chicken macrophage-like cell line HD11. After infection of HD11 cells with UEL17 and APEC O2 strains MT78 and IMT5155 (all MOI 150), caspase 3/7 activation was observed 6 hpi indicating apoptosis (Horn *et al.*, 2012). APEC may not only survive but replicate

within macrophages under certain conditions as shown after infection of HD11 cells with the highly pathogenic APEC O1 strain FY26 and APEC O2 strain CVCC249 (MOI 10; Zhuge *et al.*, 2019). In this study the APEC *acs-yjcH-actP* operon encoding the acetate assimilation system was identified as a novel intracellular survival factor promoting APEC replication within macrophages. Deletion of the *acs-yjcH-actP* operon decreased its cytotoxicity to HD11 cells as the production of pro-inflammatory cytokine mRNA IL-1β, IL-6, IL-8, IL-12β and LITAF, and iNOS production was decreased when mutant APEC strains were used. However, as APEC are facultative intracellular pathogens the importance of intracellular survival in macrophages remains unclear. Peng and colleagues showed that HD11 cells treated with APEC O78 strain 506 produced nitric oxide (NO) together with a strong increase of IL-8, IL-6, IL-1β and IL-10 mRNA expression at 18 h post-infection, while IFN-β mRNA expression remained unaffected (Peng *et al.*, 2018a). Interestingly, this response was partially dependent on the viability of APEC since stimulation with heat-killed APEC resulted in a reduced gene expression. In summary, a strong pro-inflammatory response and NO production was shown in HD11 cells which can also phagocytose and kill APEC.

The type I interferon (IFN) response after APEC infection was analysed using HD11 cells, bone marrow derived macrophages (BMDM) and primary lung macrophages (Garrido *et al.*, 2018). It was shown that priming of chicken macrophages with IFN-α increased bacterial uptake, boosted bacterial-induced ROS/NO production and augmented transcription and production of NO, IL-1β and notably IFN-β. Neutralisation of IFN-β during APEC incubation limited IFN-α-induced augmentation of the pro-inflammatory response. In the context of enhanced cellular innate immunity, Allan and colleagues showed that an innate immune cocktail of six antimicrobial host defence peptides temporarily inducing pro-inflammatory responses in innate immune cells can partially protect broiler chickens against APEC infection (Allan *et al.*, 2012).

Although the use of cell lines and *in vitro* models greatly enhance our understanding of immune responses to APEC, a recent study in chickens revealed a clear discrepancy between responses of *ex*

vivo lung macrophages and the responses *in vivo* (Alber *et al.*, 2019). Macrophages isolated from lungs and *in vitro* infected with APEC O2 harboured high numbers of bacteria in contrast to APEC O1 infected cells. *In vivo* infections with APEC O1 or O2 followed by isolation of lung cells indicated that both APEC strains were rarely observed within macrophages. It was suggested that bacterial invasion rather than phagocytosis by macrophages may be the main mechanism *in vitro* as heat-killed APEC O2 were poorly taken up by macrophages in culture similar to the *in vivo* situation. Thus, caution should be taken when (1) extrapolating *in vitro* data to the *in vivo* situation, especially when studying a facultative intracellular pathogen such as APEC and (2) assuming phagocytosis is the default mechanism while active invasion by *E. coli* may be more common which warrants to always include a killed-bacteria control in *in vitro* uptake assays.

Altogether, these studies indicate a strong cellular innate immune response early after APEC infection which inlcudes heterophils and macrophages. Further heterophil and thrombocyte studies may provide additional insights as heterophils seem to dominate the early response and thrombocytes are very abundant in the circulation. Depletion of specific immune cell types using specific antibodies or inducible-ablation using a chemical that can activate caspase expressed in transgenic chickens in a cell-specific manner could provide powerful insights into their precise role in innate responses to APEC.

The adaptive immune response

Relatively little is known about adaptive responses to APEC infection. Multiple commercial vaccines are available in the fight against colibacillosis, but their mode of action is largely unknown (reviewed in Christensen *et al.*, 2020). Historic studies focused on the role of antibodies. These studies using APEC O78 and O2 demonstrated that the initial IgM response preceded IgY (sometimes named IgG, the mammalian counterpart) several days post-vaccination of hens and showed a correlation between the hen's antibody titre and the percentage survival of the progeny after homologous APEC

challenge (Heller, 1975; Heller *et al.*, 1990). Further it was shown in chickens and turkeys that chicken egg yolk derived purified IgY or hyperimmune sera of turkeys and rabbits can protect birds against challenge with homologous APEC or closely-related serotypes using O1, O2, O78 and O35 strains, and APEC strain EC99 (Arp, 1980; Rosenberger *et al.*, 1985; Bolin & Jensen, 1987; Kariyawasam *et al.*, 2004). Rapid clearance of pathogenic APEC from the circulation of turkeys was markedly enhanced by antibody-dependent phagocytosis by liver phagocytes and to a lesser extent by splenic phagocytes (Arp, 1982). Antibodies against type I fimbriae did not enhance phagocytosis (Arp, 1985), and blood-derived pathogenic APEC strains may resist trapping and killing by macrophages in the spleen and liver (Arp & Cheville, 1981). In addition to antibody-mediated immunity early studies in turkeys and chickens also indicated increased T cell proliferation (Nakamura *et al.*, 1986; Chaffer *et al.*, 1997) as well as the lysis activity of complement which differed between different routes of vaccination indicating antibody-mediated complement lysis (Chaffer *et al.*, 1997).

More recent studies investigated the humoral and cell-mediated responses after vaccination using the Poulvac® *E. coli* vaccine, an *aroA*-deficient mutant strain of APEC serogroup O78, in more detail. Spray vaccination of broiler chicks with the Poulvac® *E. coli* vaccine indicated that after an initial innate immune response, T-cell and antibody-mediated protection may be important, as suggested by the number of $CD8^+$ cells and the production of mucosal antibodies that could be seen by the number of $CD4^+TCR2^+$ cells (Filho *et al.*, 2013). Other studies in turkeys investigated the immune response after vaccination with APEC O78 strain χ7122nal^R, Poulvac® *E. coli* vaccine or formalin-inactivated APEC O78 bacterin. Poulvac® *E. coli* and a formalin-inactivated APEC O78 bacterin conferred significant protection against homologous infection in a turkey model of acute colibacillosis (Sadeyen *et al.*, 2015b). Analysis of expression levels of signature cytokine mRNAs suggested that both vaccines induced a predominantly T_h2 response in the spleen as shown by decreased expression of *IFN-γ,* increased levels of O78-specific serum IgY and significant splenocyte recall responses to soluble APEC antigens at post-vaccination and post-challenge periods. In another

study by the same authors using the Poulvac® *E. coli* vaccine and a formalin-inactivated APEC O78 bacterin followed by a homologous APEC O78 challenge in layer chickens, an important role for antibodies was suggested as evidenced by the lack of protection in cyclophosphamide-treated B-cell depleted chickens (Sadeyen *et al.*, 2015a). Other studies exploiting bacterial outer membrane vesicles as a cross-protective vaccine candidate and a recombinant multi-antigen vaccine with broad protection potential suggested humoral and predominantly T_h1 cell-mediated adaptive immune responses in lymphoid organs (Van Goor *et al.*, 2017; Hu *et al.*, 2020).

Sadeyen and colleagues sought to define immune responses associated with clearance of sub-acute primary infections in another study in turkeys (Sadeyen *et al.*, 2014). Using a sub-acute respiratory challenge model with APEC O78 strain χ7122nal^R, cytokine, cell-mediated and humoral responses associated with protection against homologous re-challenge were shown in more detail. Levels of IL-1β and CXCLi2 mRNA expression suggested induction of a weak pro-inflammatory response in the lung, whereas a distinct anti-inflammatory cytokine profile including down-regulation of IL-1β and CXCLi2 was detected in the liver. Proliferative responses of splenocytes to either Concanavalin A or soluble χ7122nal^R antigens were negligible prior to clearance of bacteria, but APEC-specific responses were significantly elevated from 14 days post primary inoculation and at homologous secondary infection relative to control birds. Primary infection also induced significantly elevated χ7122nal^R-specific serum IgY and bile IgA responses which were bactericidal against χ7122nal^R and an isogenic Δ*rfb* mutant. Bactericidal activity was observed in the presence of immune, but not heat-inactivated immune serum, indicating that the antibodies can fix complement and are not directed solely at the lipopolysaccharide O-antigen.

Altogether, these studies indicate important roles for the humoral and cell-mediated adaptive response to APEC infection, in which the type of vaccine (and chosen adjuvant) may drive different r T_h responses. However, further studies are clearly required to elucidate the response to current and novel vaccines in more detail, as well as the serotype-specific adaptive responses to APEC.

Conclusions and outlook

Current literature on APEC as a primary pathogen has revealed a strong pro-inflammatory response early after infection in birds during which host defence peptides and the cellular innate immune response dominated by heterophils and to a lesser extent by macrophages play a role. Strikingly however, many studies both *in vivo* and *ex vivo* used high doses of APEC as inocula suggesting that birds are relatively resistant to single APEC infection. In the context of APEC dissemination in birds as well as modulation of the local immune response further immunological studies of the diverse epithelia seem warranted. Equally, relatively little is known about the adaptive immune response post-vaccination or infection with APEC and functional studies using depletion or adoptive transfer of immune system components are needed.

Overall, it is desirable to account for the vast genetic diversity of APEC serotypes, the potential for discrepancies between *ex vivo* and *in vivo* studies, and age- and breed-related differences in immune responses in future studies. Events detected with single strains, lines and sampling sites or intervals clearly may not apply universally. Moreover, it will be important to consider how APEC have evolved to modulate and evade host defences for the design of broadly effective control strategies.

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No potential conflict of interest was reported by the authors.

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