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Review article

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Abstract – Avian pathogenic *Escherichia coli* (APEC) cause aerosacculitis, polyserositis, septicemia and other mainly extraintestinal diseases in chickens, turkeys and other avian species. APEC are found in the intestinal microflora of healthy birds and most of the diseases associated with them are secondary to environmental and host predisposing factors. APEC isolates commonly belong to certain serogroups, O1, O2 and O78, and to a restricted number of clones. Several experimental models have been developed, permitting a more reliable evaluation of the pathogenicity of *E. coli* for chickens and turkeys. Hence, virulence factors identified on APEC are adhesins such as the F1 and P fimbriae, and curli, the aerobactin iron sequestering system, K1 capsule, temperature-sensitive hemagglutinin (Tsh), resistance to the bactericidal effects of serum and cytotoxic effects. Experimental infection studies have shown that the air-exchange regions of the lung and the airsacs are important sites of entry of *E. coli* into the bloodstream of birds during the initial stages of infection and that resistance to phagocytosis may be an important mechanism in the development of the disease. They have also demonstrated that F1 fimbriae are expressed in the respiratory tract, whereas P fimbriae are expressed in the internal organs of infected chickens. The role of these fimbrial adhesins in the development of disease is not yet, however, fully understood. The more recent use of genetic approaches for the identification of new virulence factors will greatly enhance our knowledge of APEC pathogenic mechanisms. Diagnosis of APEC infections is based on the clinical picture, lesions and isolation of *E. coli*. This may be strengthened by serotyping and identification of virulence factors using immunological or molecular methods such as DNA probes and PCR. Approaches for the prevention and control of APEC infections include the control of environmental contamination and environmental parameters such as humidity and ventilation. Antibiotherapy is widely used, although APEC are frequently resistant to a wide range of antibiotics. Vaccines containing killed or attenuated virulent bacteria protect against infection with the homologous strain but are less efficient against heterologous strains. Hence, vaccination for colibacillosis is not widely practised because of the large variety of serogroups involved in field outbreaks. © Inra/Elsevier, Paris.

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Résumé – *Escherichia coli* **pathogènes aviaires (APEC).** Les *Escherichia coli* pathogènes aviaires (ou APEC) sont responsables d'aérosacculite, de lésions fibrineuses des séreuses, de septicémie et d'autres pathologies extra-intestinales chez le poulet, la dinde et d'autres espèces de volailles. Les APEC sont présents dans la flore intestinale des oiseaux sains et la plupart des pathologies qui leur sont associées sont secondaires à l'action de facteurs prédisposants. Les APEC appartiennent fréquemment à trois sérogroupes : O1, O2 et O78, ainsi qu'à un nombre limité de clones. Plusieurs modèles d'infection expérimentale permettent une évaluation fiable de la virulence pour le poulet et la dinde. Les facteurs de virulence identifiés chez les APEC comprennent des adhésines (fimbriae F1, P et curli), le système aérobactine d'acquisition du fer, la capsule K1, l'hémagglutinine thermosensible Tsh, la résistance au pouvoir bactéricide du sérum, ainsi qu'un pouvoir cytotoxique. Des infections expérimentales chez le poulet ont mis en évidence que les zones d'échanges gazeux, sacs aériens et poumons, constituaient des sites de pénétration des APEC dans la circulation sanguine. La résistance à la phagocytose serait un autre mécanisme important dans le développement de l'infection. Il a été démontré également que les fimbriae F1 ne sont exprimés que dans le tractus respiratoire, alors que les fimbriae P sont exprimés dans les organes internes des poulets infectés. Le rôle de ces adhésines dans la pathogénie n'est pas complètement élucidé. L'identification de nouveaux facteurs de virulence par les approches génétiques en cours devrait permettre d'accroître les connaissances sur les APEC. Le diagnostic de colibacillose repose habituellement sur le tableau clinique, l'observation de lésions caractéristiques et l'isolement de *E.coli*. Il peut être confirmé par la détermination du sérotype et la détection de facteurs de virulence par des méthodes immunologiques ou moléculaires (PCR, sondes ADN). Dans l'élevage, le contrôle de la contamination de l'environnement ainsi que des paramètres tels que la température et l'humidité constituent des méthodes de prévention de la colibacillose aviaire. Les traitements antibiotiques sont largement utilisés bien que les APEC soient fréquemment résistants à plusieurs antibiotiques. Des vaccins à base de bactéries tuées ou à virulence atténuée peuvent protéger contre la souche homologue d'*E.coli*, mais sont peu efficaces contre des souches hétérologues. De fait, la vaccination contre la colibacillose est peu utilisée en raison de la grande diversité des sérotypes d'*E. coli* qui peuvent être impliqués. © Inra/Elsevier, Paris.

Escherichia coli / aviaire / virulence / fimbriae / capsule / aérobactine

Plan

1. INTRODUCTION

Although *Escherichia coli* is present in the normal microflora of the intestinal tract and other host mucosal surfaces and in the bird's environment, only certain of these strains possessing specific virulence attributes, designated as avian pathogenic *E. coli* (APEC), are able to cause disease. APEC are mostly associated with extra-intestinal infections, principally of the respiratory tract or systemic infections, and result in a variety of diseases which are responsible for severe economic losses [46]. Most of the diseases associated with APEC are secondary to environmental and host predisposing factors. Therefore, losses due to these diseases may be greatly reduced by controlling these factors. In the last several years, the increasing use of more sophisticated molecular approaches for the study of bacterial pathogenesis have led to some exciting insights into the virulence determinants of APEC and the mechanisms by which these bacteria are able to develop infection and cause disease, although the pathogenic mechanisms of APEC have not yet been fully elucidated. In this chapter, we will highlight the recent advances in this area, and approaches currently being undertaken to further our knowledge.

2. DISEASE SYNDROMES ASSOCIATED WITH APEC

APEC are mostly associated with infection of extraintestinal tissues in chickens, turkeys, ducks and other avian species with the exception of a possible relationship with the development of enteritis [44, 46]. Yolk sac infections are most frequently observed towards the end of the egg incubating period, usually following fecal contamination of the egg surface. They often result in embryonic mortality or death of the young birds for up to 3 weeks following hatching. Retention of the infected yolk sac and omphalitis are often observed. The most important dis-ease syndrome associated with APEC begins as a respiratory tract infection and may be referred to as aerosacculitis or the airsac disease. If unchecked, this infection may evolve into a bacteriemia and a generalized infection which manifests as a polyserositis. The respiratory tract complex is most often observed in birds of 4 to 9 weeks of age and may result in extensive economic losses with up to 20 % mortality as well as reduced growth and feed efficiency and an increased condemnation rate at the abattoirs. APEC infection of the respiratory tract is secondary to initial infection with one or more of the respiratory tract agents: Newcastle disease virus (NDV), infectious bronchitis virus (IBV) and *Mycoplasma gallisepticum,* or with the NDV or IBV vaccine viruses [74, 75, 98]. Susceptibility of birds to APEC infection is increased by deciliation of the epithelial cells of the upper respiratory tract following exposure to ammonia and dust in the immediate environment of the birds. Respiratory tract infection with APEC results in depression, fever and death. Respiratory lesions include aerosacculitis with a serous to fibrinous exudate, an initial infiltration with heterophils and a subsequent predominance of mononuclear phagocytes. In more generalized infections, lesions of pericarditis and perihepatitis are also observed. In adult birds, an acute form of septicemia due to APEC may occur. Lesions may be absent or include pericarditis, peritonitis and bile-staining and necrotic foci in the liver. In laying birds, APEC may infect the oviduct via the left abdominal airsac leading to salpingitis and loss of egg laying ability. Alternatively, APEC may sporadically invade the peritoneal cavity via the oviduct leading to peritonitis and death. In broilers, broiler breeders and layers, APEC may cause a specific syndrome called the swollen head syndrome, following an initial infection with turkey rhinotracheitis virus [64] or possibly other viruses such as IBV or NDV. Lesions observed in this syndrome include cellulitis and oedema of the facial skin and periorbital tissues. In broilers,

APEC are also associated with a cellulitis or necrotic dermatitis of the lower abdomen and thighs [33, 41, 71]. This disease has been reported more and more frequently in recent years and although it does not cause clinical signs or mortalities, the associated subcutaneous fibrinous lesions result in extensive economical losses due to condemnation of carcasses [34].

APEC are probably not a cause of intestinal diseases such as enteritis in poultry, although enterotoxigenic *E. coli* have occasionally been associated with outbreaks of diarrhea in poultry [4, 59].

3. EPIDEMIOLOGY OF APEC INFECTIONS

E. coli are normal inhabitants of the lower digestive tract of many avian species, and 10^4–10^7 colony forming units (cfu) per gram are usually present in the intestinal contents of birds. *E. coli* also colonize the upper respiratory tract (pharynx and trachea), and can be isolated from skin and feathers, depending on the level of environmental contamination [53]. Pathogenic as well as nonpathogenic *E. coli* isolates can be recovered at these sites from healthy birds.

The contamination of birds with *E. coli* occurs in the first hours following hatching, and *E. coli* strains rapidly multiply in the intestine. Many different strains can be acquired during the life of a bird. Vertical contamination results from the transmission of *E. coli* from breeders, via contaminated shells during hatching, or in ovo, as a result of salpingitis [46].

Horizontal contamination with *E. coli* usually occurs through contact with other birds, or through feces, contaminated water and feed. Birds are frequently contaminated by inhalation of particles present in dust which can contain as many as 10^6 cfu of bacteria per gram. Carlson and Whenham [17] have demonstrated that the risk of colibacillosis increases with the level of environmental contamination.

4. CHARACTERIZATION OF APEC

4.1. Serogroups

APEC isolates commonly belong to certain O serogroups, particularly 1, 2, 8, 15, 18, 35, 78, 88, 109 and 115 [46]. As first demonstrated by Sojka and Carnaghan [99], three of these, O1, O2 and O78 are the most frequently recovered from colibacillosis in the different countries worldwide, and they represent 15–61 % of the total number of isolates, depending on the study [13, 20, 29, 40]. However, many pathogenic isolates do not belong to these identified pathogenic serogroups, and they are commonly designated as 'untypable'. This results in difficulties in identifying APEC strains in veterinary laboratories, because at present diagnosis mainly relies on serogrouping.

4.2. Biochemical properties

Several biochemical characteristics have been associated with APEC, such as the fermentation of dulcitol or of salicin, but, in fact, this represents more a correlation with the serogroup of the strains rather than with their virulence. An example is the positive correlation between the fermentation of adonitol by O35 *E. coli* strains that were responsible for numerous cases of colibacillosis in Delaware (USA) between 1981 and 1983 [20].

4.3. Clonal relationships

The molecular characterization of APEC strains, which permits the evaluation of genetic relatedness, has been widely documented. Classification of isolates is based on electrophoretic types (ET), as defined by electrophoretic detection of allelic variants of enzyme-coding genes. Certain APEC strains isolated from different countries and at different times are genetically related and belong to the same clone [114]. Further-

more, it has been demonstrated that some clones are specific to APEC: in a comparison of 45 *E. coli* isolates from poultry, White et al. [115] showed that 83 % of pathogenic isolates belong to only five clones, whereas each of ten non-pathogenic strains belong to different clones. Similar results have been obtained by other methods such as ribotyping (Coulange et al., unpublished results). It is noteworthy that some APEC strains belong to the same clones as do pathogenic *E. coli* isolated from extra-intestinal infections in humans [114].

4.4. Experimental testing of APEC in animals

Several experimental models have been developed, allowing the evaluation of the pathogenicity of *E. coli* for chickens or turkeys. Pathogenic *E. coli* isolates are able to kill embryos or 1-day-old chicks following subcutaneous inoculation [25, 52]. Both of these models give rapid results and permit the measurement of the virulence of the isolates according to their 50 % lethal dose. However, they bypass the natural route of infection by avoiding the respiratory tract. Birds can also be inoculated intravenously [97].

Other experimental models which reproduce natural disease in birds at susceptible ages that correspond to those of field disease (2–4 weeks old) have also been used. Bacteria can be aerosolized [43], inoculated in the naso-pharynx [98], or directly inoculated into the trachea [15, 42], following a preliminary challenge with a triggering agent such as a virus (infectious bronchitis virus, or Newcastle virus), mycoplasma, or an increase in ammonia which impairs the natural defences of the respiratory tract. Typical lesions of colibacillosis are thus reproduced and several criteria, such as weight gain, presence of fibrin on airsacs, lesions of pericarditis and perihepatitis, and contamination of internal organs and blood can be recorded. This permits confirmation of the pathogenicity of the tested strain.

The direct inoculation of *E. coli* into the airsacs is a 'high performance' model as it does not require the preliminary action of triggering agents, and as it results in typical lesions of colibacillosis with greater homogeneity in bird responses, as compared to inoculations in the upper respiratory tract [87, 89].

In all these models, the use of specific pathogen-free (SPF) birds is a necessary condition to avoid cross contamination with other pathogens. In some cases, axenic chickens have been used. This approach has allowed the visualization of the inoculated *E. coli* strain in the contaminated tissues without the interference of commensal *E. coli* strains [30].

5. VIRULENCE FACTORS OF APEC

Several potential virulence factors have been identified on APEC, mainly from a positive correlation between phenotypic characteristics and virulence for chickens. The study of the involvement of these factors in virulence using experimental models of infection is just beginning. This involvement includes the adherence ability of bacteria to the respiratory tract, mediated by fimbriae, the resistance of bacteria to the immunological defences, the multiplication of bacteria in the host physiological liquids through the expression of iron siderophores, and the ability to produce cytopathic effects. More recently, genomic methods have been used, providing very interesting additional hypotheses.

5.1. Fimbriae

5.1.1. F1 fimbriae (type 1)

Evidence that the ability of *E. coli* to adhere to the epithelium of the respiratory tract of chickens could be a virulence factor

was first provided by the observation that a virulent and fimbriated strain was less easily cleared from the trachea of turkeys than an avirulent and less fimbriated strain [8]. These results were strengthened by the demonstration that virulent *E. coli* strains were better colonizers of the chicken trachea than avirulent strains, and that these adhesive properties were mediated by type 1 fimbriae [24, 26]. Adhesion of type 1 fimbriae to chicken epithelial cells of the pharynx and trachea was demonstrated both in vivo and in vitro [25, 76]. Gyimah and Panigrahy [49] blocked the specific adherence of APEC strains to chicken tracheal sections with specific anti-type 1 fimbriae serum and by D-mannose which is the cellular receptor of the adhesin of type 1 fimbriae.

Type 1 fimbriae consist of a major protein, FimA, associated with ancillary proteins, FimF, FimG and the adhesin FimH. They are encoded by the *fim* gene cluster, which is located at 98 mn on the chromosome of *E. coli*, and comprises nine genes, seven of which are present in a single operon whose expression is controlled by an invertible element containing the promoter [85].

The presence of type 1 fimbriae is more frequent on pathogenic than on nonpathogenic avian *E. coli* strains, even though these fimbriae are common among *E. coli*. Dozois et al. [29] demonstrated the presence of the *fimD* gene in 74 % of the 112 APEC isolates but only in 55 % of the *E. coli* isolated from healthy birds. Wooley et al. [116] found that 100 % of the APEC strains produced type 1 fimbriae as compared with 57.5 % of the commensal strains.

Several variants of type 1 fimbriae have been described on APEC and they seem to be related to the serogroup of the strains [27, 102]. They differ from classical type 1A fimbriae with respect to the molecular weight of the major fimbrial subunit and its immunological reactivity. More recently, four variable regions have been identified in the *fimA* gene of an APEC isolate, among which at least two regions could be specific

for isolates of the O2 serogroup [68]. The FimH adhesin is located at the tip of the fimbriae, or both at the tip and along the fimbriae, depending on the APEC strain [18]. The significance of these different locations is unknown.

In vivo, type 1 fimbriae are expressed mainly in the trachea and in the lungs and airsacs, but their expression has never been observed in other organs nor in the blood [30, 91]. This could result from the phase variation of type 1 fimbriae, depending on the in vivo environmental conditions.

The role of type 1 fimbriae in infection is unclear. By using a mutant deleted for the entire *fim* operon, Marc et al. [69] showed that the expression of type 1 fimbriae is not necessary for the colonization of the trachea and airsacs, but that these fimbriae could play a role in the colonization of the lungs.

Type 1 fimbriae could also play a role in the interaction of APEC strains with the immune system, although this role is controversial. Orndorff et al. [85], suggested that type 1 fimbriae could protect *E. coli* from phagocytosis. Other studies demonstrated that the resistance to the bactericidal effects of serum was positively correlated with the presence of type 1 fimbriae [29, 116]. It was recently demonstrated that type 1 fimbriae could be mastocyte activators via the FimH adhesin, and that this activation would result in phagocytosis of bacteria and recruitment of neutrophils at the site of infection [65–67]. Furthermore, Pourbakhsh et al. [90] showed that highly virulent APEC strains were resistant to the bactericidal effect of macrophages when they did not express type 1 fimbriae, but were sensitive when they did express these fimbriae.

Further studies are needed to clarify the role of type 1 fimbriae in the virulence of APEC as a favorable or unfavorable factor. This topic is also presently controversial for other extraintestinal pathogenic *E. coli*, such as those responsible for urinary tract infections.

5.1.2. P fimbriae

P fimbrial adhesins were initially found on *E. coli* associated with upper urinary tract infections in humans. They mediate bacterial adherence to uroepithelial cells and are an important virulence determinant in the development of pyelonephritis [60]. P fimbriae are encoded by the *pap* gene cluster, which is chromosomally located and consists of 11 genes whose structure and function have been extensively studied [51]. P fimbriae consist of a major fimbrial subunit, PapA, and a terminally located fimbrial adhesin, PapG. At least 11 serotypes of P fimbriae, F7–F16, have been recognized based on antigenic differences in the major fimbrial subunits [51]. Receptor specificity of P fimbriae is conferred by the adhesin PapG, for which three variants, classes I, II and III have been identified [101]. These variants recognize different isoreceptors of the globoseries of glycolipids, which contain the disaccharide gal-gal and may also be distinguished by their mannose-resistant hemagglutination (MRHA) of different erythrocytes.

Certain APEC strains also express P fimbrial adhesins [1, 29, 31, 108, 110]. In general, these adhesins have been observed in a low proportion of the isolates studied. Achtmann et al. [1] found that 52 % of the O2 isolates from septicemic chickens expressed P fimbrial adhesins as detected by MRHA. Dozois et al. [29, 31], however, observed the expression of P fimbrial adhesins, as detected by MRHA and immunofluorescence, only in O1 isolates from septicemic turkeys and in an O18 isolate from a septicemic chicken, in a study of 112 *E. coli* isolates from chickens and turkeys with septicemia. The P fimbrial adhesin from one of the O1 isolates was shown to be closely related to F11 fimbriae associated with *E. coli* isolated from upper urinary tract infections in humans, by N-terminal amino acid sequencing, immunoblotting, and competitive ELISA [88]. Van den Bosch et al. [108] reported that 78 % of 203 *E. coli* isolates

from chickens with septicemia, or 96 % (when only isolates of serotypes O1: K1, O2: K1, O35 and O78: K80 were considered) expressed P fimbrial adhesins of serotype F11, as detected by ELISA.

It is interesting to note that *pap*-related DNA sequences were observed in a much higher proportion in *E. coli* isolates in the study by Dozois et al. [29]. It was found that 44 and 31 % of 81 and 29 isolates from septicemic chickens and turkeys, respectively, possessed *pap*-related DNA sequences. The presence of *pap*-related DNA sequences was significantly more frequent in isolates from septicemic than from healthy chickens. In isolates from septicemic turkeys, their presence was also associated with lethality in 1-day-old chicks. Although *pap*-related DNA sequences were present in isolates of serogroups O1, O2 and O78, the in vitro expression of the P fimbrial adhesins was only observed in O1 and O18 isolates, even following growth of the bacteria in culture conditions optimal for the production of these fimbrial adhesins [31]. Further examination of these isolates by PCR and Southern blot hybridization [32] demonstrated that only those isolates expressing the P fimbrial adhesin possessed a complete copy of the *fel* gene cluster which encodes P fimbrial adhesins of the F11 serotype. In contrast, the isolates not expressing the P fimbrial adhesin, mainly isolates of the O78 serogroup, possessed partial or divergent P fimbrial clusters, which explained their inability to express these fimbriae.

The role of P fimbrial adhesins in the pathogenicity of APEC has not yet been fully elucidated. These adhesins do not appear to be involved in bacterial adherence to chicken tracheal or pharyngeal cells in vitro [108, 110], nor to frozen sections of the chicken trachea [30], suggesting that receptors for these adhesins are not present at this site. Pourbakhsh et al. [90, 91] demonstrated that chickens inoculated with an F11 P-fimbriated APEC strain by the intratracheal or airsac route mounted a spe-

cific anti-F11 antibody response as revealed by ELISA, providing evidence that these fimbriae are produced in vivo. No expression of this fimbrial adhesin was observed in bacteria colonizing the trachea of inoculated chickens, as detected by immunofluorescence, whereas bacteria colonizing the airsacs, lungs and internal organs of these same chickens expressed P fimbriae. These results provide strong evidence for in vivo phase variation of P fimbrial adhesins with respect to their localization in the host and reinforce the suggestion that P fimbrial adhesins are not important in the initial colonization of the upper respiratory tract but in the later stages of the infection.

5.1.3. Curli

Curli are thin, coiled filamentous structures of about 2 nm in diameter found on the bacterial surface of *E. coli* and *Salmonella* spp. [21, 81]. These structures mediate bacterial binding to extracellular matrix and serum proteins such as fibronectin, laminin, plasminogen and plasminogen activator protein [81]. Curli are optimally expressed in vitro at 26 °C in a stationary phase and in a low osmolarity growth medium [82].

Initially, it was thought that curli were encoded by the *crl* gene [81] although it was subsequently shown that *crl* plays a role in but is not necessary for, the expression of the curli phenotype in an APEC strain [92]. In fact, *crl* activates cryptic genes for curli formation [6]. It has more recently been shown that the *csgA* (curlin subunit gene) encodes for the major curlin subunit [83].

It appears that most *E. coli* strains carry the *csgA* gene, although the curli are not always expressed in in vitro growth conditions [83]. Maurer et al. [70] showed that the *csgA* gene was present in all of the 78 *E. coli* isolates from diseased birds and also in all of the 50 commensal *E. coli* isolates from healthy chickens. Similarly, Dho-Moulin et al. [28] showed that 298 of the 300 *E. coli* from diseased birds possessed the *csgA* gene.

In addition, Maurer et al. [70] found that only half of the examined isolates produced the curli following bacterial growth in culture conditions optimal for curli expression.

The role, if any, of curli in the pathogenic process has not yet been elucidated. Nevertheless, certain properties of curli, such as the ability to bind to the major histocompatibility complex class I molecules which are present on most cells of higher vertebrates [84], or the ability to bind to the extracellular matrix and serum proteins [81], may contribute to bacterial adherence and colonization in the initial stages of infection.

5.2. Aerobactin iron-sequestering system

The low concentration of free iron in physiological liquids of animals (about 10^{-18} mol.L^{-1}) is not sufficient to allow bacterial growth which requires a concentration of about 10^{-6} mol.L^{-1}. Numerous pathogenic bacteria with invasive abilities have developed high affinity iron-acquisition systems which can compete with the host siderophores such as transferrin, and thus favor bacterial growth in low iron environments.

Dho and Lafont [25] found a positive correlation between the ability of avian *E. coli* strains to grow in vitro under iron-limiting conditions and the lethality for 1-day-old chicks. They subsequently demonstrated that this was due to the expression of the aerobactin system [62]. Several studies have confirmed that most APEC strains (73–98 %) possess and express the aerobactin iron-acquisition system, whereas non-pathogenic strains produce aerobactin far less frequently [29, 36, 63]. The high correlation observed between the presence of the aerobactin system and the virulence of APEC has recently lead to the use of diagnostic tests based on the immunological detection of the IutA protein that is the receptor for the ferric aerobactin.

The role of the aerobactin system in the multiplication of APEC in extraintestinal locations during infection can be highly suspected. The aerobactin system is expressed in vivo as shown by the detection of anti-aerobactin antibodies, following intra-tracheal inoculation of axenic chickens (Brée, pers. comm.).

The aerobactin operon is usually carried by large colV plasmids, which are of at least 80 kb [107, 111]. In some cases, the presence of large plasmids has been related to APEC virulence [96,109]. Ike et al. [56] demonstrated that the loss of a large conjugative colV plasmid of an O2 APEC isolate resulted in a reduction in virulence, in the loss of resistance to the bactericidal effects of serum, and in the loss of the aerobactin system. The reintroduction of the plasmid into the parent strain restored these three properties. These results support the idea that the large plasmids of APEC may encode several virulence determinants. The presence of the *iss* gene on the plasmid colV-IK94, and of the *traT* gene on drug resistance plasmids, were shown to be correlated with increased survival in serum and enhanced virulence of the strains carrying these plasmids [3, 9, 10, 72].

5.3. Capsule

Some polysaccharidic capsules of *E. coli*, especially those containing N-acetyl neuraminidic acid, are able to interact with C3 to C3b activators in the classical and alternative complement pathways. This induces resistance of the bacteria to the bactericidal effects of the complement [58]. The K1 antigen is frequently associated with APEC of the more pathogenic serogroups such as O1 and O2, and it is also often present on nontypable APEC strains [46].

The K1 antigen is poorly immunogenic, and could thus be involved in the resistance of APEC to the immunological defences of the bird. This hypothesis is strengthened by the observation that K1 mutants of human pathogenic *E. coli* strains were far less lethal for 1-day-old chicks than the wild-type strain [23]. More recently, Pourbakhsh et al. [90] showed that three APEC strains possessing the K1 antigen were more resistant to the bactericidal effects of serum than three other APEC strains expressing different K antigens.

5.4. Temperature-sensitive hemagglutinin

A hemagglutinating activity, preferentially expressed at low temperatures (26–30 °C) and repressed at 42 °C, was observed on an APEC strain by Provence and Curtiss III [93]. This phenomenon was called 'temperature sensitive hemagglutination'. The *tsh* gene was cloned from this strain, and it was deduced from its sequence that it encodes a protein of 140 kDa, with a mature form of 118 kDa. The protein Tsh sequence showed homologies with immunoglobulin A proteases from *Haemophilus influenzae* and *Neisseria gonorrhoeae*, as well as with the SepA protein from *Shigella flexneri*, and with the EspC and EspP proteins which are present on EPEC and EHEC strains. Neither the proteolytic activity of Tsh for A immunoglobulins, nor the activity of SepA, EspB and EspP are presently known, and the observed similarities between Tsh and these proteins may result from similar secretory systems.

Maurer et al. [70] have demonstrated that the *tsh* gene was present on 46 % of clinical *E. coli* isolates of avian origin, but none were found in isolates from healthy animals. In another study of 300 avian *E. coli* originating from France and Quebec, Dho-Moulin et al. [28] showed that among *tsh* positive isolates, the incidence of pathogenic isolates (90.6 %) was far higher than that of non-pathogenic isolates. This suggests that the Tsh protein could play a role in the pathogenic process, although its precise function has not yet been elucidated.

5.5. Resistance to the bactericidal effects of serum

Resistance to the bactericidal effects of the complement in serum, mediated by bacterial surface structures such as capsule, lipopolysaccharide, ColV production and outer membrane proteins, has been associated with APEC isolates, particularly those originating from septicemic birds [46, 77, 117]. For example, Ellis et al. [35] demonstrated a correlation between serum resistance and virulence for intravenously inoculated 3-week-old turkeys, in *E. coli* isolates from turkeys. Dozois et al. [29] observed that serum resistance was associated with isolates from septicemic turkeys and with lethality in isolates from septicemic chickens, in a study of 175 *E. coli* isolates from septicemic and healthy birds. Ike et al. [56] also found a strong correlation between serum sensitivity and lethality for 1-day-old chicks, in 115 *E. coli* isolates from septicemic chickens. Wooley [116] found a strong association between serum resistance and isolates from septicemic chickens, in a study of 80 *E. coli* isolates from septicemic and healthy chickens. Nolan et al. [78] demonstrated a high correlation between serum resistance and lethality for 21-day-old chickens. In order to investigate the role of complement resistance in the virulence, Nolan et al. [79] produced an avirulent, complement-sensitive mutant from a virulent, complement-resistant APEC isolate. This mutant possessed a 16.2-kDa outer membrane protein (OMP) not present in the wild-type strain, suggesting that an as yet unidentified OMP may be responsible for the complement resistance of this APEC isolate [80]. Further characterization of this mutant [61] suggested that the complement resistance of this isolate is due, at least in part, to its ability to restrict C3 deposition, but not to degrade C3, on the bacterial surface.

5.6. Toxins and cytotoxins

There are few reports demonstrating that APEC are able to produce toxins that may be involved in the pathogenic process. Early studies have suggested that some APEC might produce exotoxins such as the chick-lethal toxin (CLT) [105], although production of this toxin did not seem to be widespread in APEC. Subsequently, Emery et al. [36] demonstrated that in up to 22.5 % of 500 *E. coli* from colisepticemic chickens or turkeys, two distinct heat-labile toxins (LT) with cytotoxic activity for Y-1 and/or Vero cells were produced. Fantinatti et al. [37] observed a cytotoxic activity for Vero cells only in three of the most pathogenic isolates in a study of 17 isolates from septicemic chickens. More recently, Blanco et al. [12] found that only 7 % of 645 *E. coli* isolates from septicemic or healthy chickens were toxigenic, producing a cytotoxic response in HeLa but not in Vero cells, an enterohemolysin, or a new cytotonic product in HeLa and Vero cells. This toxigenicity did not appear to be related to septicemic isolates. No isolates producing enterotoxins STa or LT, verotoxins VT1, VT2 or VT2v, or cytotoxic necrotizing factors CNF1 or CNF2 were detected in this study. Similarly, Irwin et al. [57] did not find any vero-toxin-producing *E. coli* in cloacal samples from 500 broiler chickens.

Parreira and Yano [86] demonstrated the production of a cytotoxin active on Vero and HeLa cells in 72 % of 50 isolates taken from chickens with the swollen head syndrome. They have designated this toxin VT2y because of the similarity of its effect to that of the verotoxins and the neutralization of its effects by antiserum against VT2. However, in the conditions of stringency used in this study, DNA probes for VT1 and VT2 did not hybridize with VT2y-positive isolates. Nevertheless, the production of a verotoxin, which targets vascular endothelium, would be consistent with the lesion of oedema observed in affected birds.

Enterotoxigenic *E. coli* have occasionally been isolated from the intestines of chickens with diarrhea. For example, Akashi et al. [4] detected the genes for STII (STb) in seven of 38 *E. coli* isolates from fecal

samples of chickens with diarrhea in the Philippines. In another study of enterotoxigenic *E. coli* isolates from chickens with diarrhea in the Philippines [106], an LT-like enterotoxin similar to LTp with respect to antigenicity and amino acid composition was identified.

5.7. Approaches for the identification of novel virulence factors

In addition to the characterization of putative virulence genes and the study of their role in virulence, novel approaches have been recently undertaken to identify genomic regions specific for APEC strains, which could be included in pathogenicity islands.

Brown and Curtiss III [16] performed a genomic subtraction between an APEC isolate (serogroup O78) and a K12 *E. coli*. This enabled them to identify and locate 12 unique regions on the chromosome of the APEC isolate. Five of these unique regions corresponded to the positions of previously reported virulence factors such as the *tsh* gene, the group II capsule genes, the *rfb* gene cluster, and the pathogenicity islands PAI I (LEE) and PAI II. By using a similar approach, Coulange [22] isolated 17 fragments specific for a pathogenic strain after a subtractive hybridization between a pathogenic and a non-pathogenic avian *E. coli* strain of serogroup O2. In a collection of 67 avian *E. coli* isolates, nine of these fragments were more frequent among pathogenic than among non-pathogenic isolates. The construction of mutants in these regions will help to understand the role they play in virulence.

The use of genetic approaches for the identification of new virulence factors will greatly improve our knowledge of APEC pathogenic mechanisms. The application of methods such as signature-tagged mutagenesis [55] (allowing the identification of genes which are expressed in vivo by a negative selection) or arbitrarily primed PCR [113] (identifying specific mRNA produced by a pathogenic strain) could provide valuable information in the study of APEC.

6. PATHOGENESIS OF APEC INFECTIONS

In the last several years, our understanding of how the lesions of diseases due to APEC develop and of the mechanisms by which APEC are able to cause these lesions has greatly increased. This is particularly the case for the respiratory tract complex, and this will be the focus of this review. Natural respiratory tract infection of poultry by APEC is thought to occur via the inhalation of feces-contaminated dust [46]. Clearance of inhaled particles in the avian lung seems mainly to be through phagocytosis by atrial and infundibular epithelial cells of the parabronchial region, as there is no known cellular defence similar to the mammalian aveolar macrophage in the gas-exchange area [100]. Similarly, the avian airsac has no known resident cellular defence mechanisms and must rely on an inflammatory influx of heterophils as the first line of cellular defence [38, 39, 103, 104]. Hence, the air-exchange regions of the lung and airsacs are rather vulnerable to bacterial colonization and invasion. It has been shown that the air-capillary region of the lung is an important site of entry of *E. coli* into the bloodstream of birds [2, 19, 89, 95]. Pourbakhsh et al. [89] also observed bacteria adhering to and within the epithelial cells, in the interstitium, and in the lumen of airsacs and within vascular endothelial cells in chickens following airsac inoculation with an APEC isolate. These results indicate that the passage of APEC across the airsac barrier is also a site of entry into the bloodstream early in infection.

In order to more fully understand the virulence mechanisms of APEC, Pourbakhsh et al. [90] examined the dynamics of infection in chickens following inoculation by the air-

sac route with *E. coli* isolates of high or low pathogenicity. At 6 h postinoculation, all isolates had colonized the respiratory tract and internal organs, but bacteria were recovered from the pericardial fluid and blood only in the highly pathogenic isolates. Apparently viable bacteria of the highly pathogenic isolates, but not the low pathogenic isolates, were often observed to be associated with or within macrophages in the airsacs and lungs of inoculated birds. A strong correlation was also observed between pathogenicity for chickens in vivo and the ability to resist the bactericidal effects of chicken macrophages in vitro. These results suggest that the resistance to phagocytosis may be an important mechanism in the development of avian colisepticemia.

7. DIAGNOSIS, PREVENTION AND CONTROL OF APEC INFECTIONS

7.1. Diagnosis

A diagnosis of colibacillosis is first suggested by the clinical picture and by the presence of typical macroscopic lesions such as airsacculitis, sometimes associated with pericarditis and perihepatitis. These lesions can, however, also be caused by other organisms. Airsacculitis can be caused by *Mycoplasma* and *Chlamydia*, pericarditis can also be caused by *Chlamydia*, and perihepatitis is sometimes caused by *Pasteurella, Salmonella* or other organisms [44]. Thus, in the presence of lesions evoking colibacillosis, the diagnosis has to be confirmed by the isolation of pathogenic *E. coli*. Cultures should be taken from the heart blood and affected tissues, such as the liver, spleen, pericardial sac and marrow, avoiding contamination with the intestinal contents. The isolation of *E. coli* should be verified by using appropriate media (McConkey agar, eosin-methylene blue agar or drigalki agar),

and based on biochemical reactions. The indicators used for the identification of *E. coli* comprise indole production, fermentation of glucose with gas, presence of a β-galactosidase, lack of production of hydrogen sulfide and of urease, and the non-utilization of citrate as a carbon source. This diagnosis is strengthened if the isolated culture belongs to a known pathogenic serogroup (O1, O2, O78) and/or expresses the aerobactin system. The presence of other virulence factors, such as P fimbriae, the K1 capsule and Tsh protein (although these factors are not present in a high proportion in pathogenic strains, they are very infrequent in non-pathogenic strains), may help to confirm the identification of the isolate as an APEC.

Serotyping and detection of the aerobactin system can be performed using immunological methods. Other virulence factors are best detected by molecular methods such as specific PCR assays or the use of specific DNA probes.

7.2. Prevention

The disease can be prevented by controlling environmental contamination in order to avoid predisposing respiratory infections. The most direct method would be to reduce and to control intestinal contamination by pathogenic serogroups. Weinack et al. [112] found that pathogenic serotypes could be competitively excluded from the intestinal tract by seeding newly hatched chicks with the intestinal flora of resistant chickens. Other preventive methods include the reduction of the transmission of *E. coli* by fumigating the eggs within 2 h after they have been laid and by discarding eggs that are cracked or those with obvious fecal contamination. Infection of the respiratory tract of birds can be reduced by maintaining mycoplasma-free birds, and by controlling the environmental parameters (humidity, ventilation, dust and ammoniac in the air).

7.3. Control

Presently, the treatment of colibacillosis relies mainly on antibiotherapy. As a high proportion of pathogenic isolates of *E. coli* from poultry are resistant to numerous antibiotics [5, 11, 94], isolates should be tested for antibioresistance before treatment. The most frequently used antibiotics are quinolones, beta-lactamines, tetracyclines and sulfamides [46]. Treatment with substances that increase the effectiveness of phagocytes, such as ascorbic acid, corticosterone and deoxycorticosterone, has also been suggested [45, 47, 48].

Various vaccines which employed killed or attenuated virulent bacteria have been experimentally tested. They generally confer a good protection against infection with the homologous strain, but cross-immunity against heterologous *E. coli* strains is not as efficacious. Similarly, passive immunization of young birds is satisfactory when birds are further challenged with the homologous strain. Heller et al. [54] showed that breeders immunized with vaccines containing sonicated bacteria harbored detectable antibody titers for several months, and that passive immunity of their chicks against the homologous *E. coli* strain was completely protective for 2 weeks. Passive immunity results in increased clearance of bacteria from the blood, spleen, liver and lungs [7, 73]. Vaccines against *E. coli* are not widely employed, probably because of the large variety of serogroups involved in field outbreaks.

Experimental assays have attempted to use virulence factors as antigens for vaccination. Highly purified pilus vaccines proved to be effective against infection with bacteria possessing the appropriate pili [50]. Bolin and Jensen [14] passively immunized 18-day-old turkeys with a rabbit antiserum prepared against outer membrane preparations of *E. coli* grown in iron-limiting conditions. When challenged into the airsacs with homologous strain, immunized turkeys were partially protected against infection.

8. CONCLUSION

Recent advances in the study of the virulence factors of APEC have resulted in a greater understanding of the mechanisms by which these bacteria are able to develop infection and cause disease. F1 fimbriae appear to be involved predominantly in the initial bacterial colonization of the upper respiratory tract. Subsequent multiplication and persistence of the bacteria following invasion of the host would be enhanced by possession of the aerobactin system and resistance to the non-specific immune defences of the host by such bacterial attributes as the presence of the K1 capsule and phase variation of F1 fimbriae. P fimbrial adhesins may also have a role at this level, since they are expressed exclusively in internal organs. Their role in the infection process, as well as that of Tsh and curli, is yet to be elucidated. Entry of bacteria into the bloodstream from the respiratory tract appears to occur in the air-exchange regions of the lungs and the airsacs. However, the bacterial mechanisms for this entry and for resistance to the phagocytic defences of the host upon entry, which appears to be an important attribute of highly pathogenic strains, remain unknown. Since many APEC lack the known virulence factors, it is reasonable to suppose that additional as yet uncharacterized factors, possibly expressed only within the host, exist. The current use of molecular approaches such as subtractive hybridization combined with infection studies in well-defined natural host experimental models using isogenic mutant strains should lead to some exciting insights into the pathogenic mechanisms of APEC in the near future.

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