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Reviews

Avian pathogenic Escherichia coli - methods for improved diagnosis

H.C. KUNERT FILHO, D. CARVALHO, T.T. GRASSOTTI, B.D. SOARES, J.M. ROSSATO, A.C. CUNHA, K.C.T. BRITO, L.S. CAVALLI and B.G. BRITO*

FEPAGRO Animal Health – Institute of Veterinary Research 'Desidério Finamor' (IPVDF), Avian Health Laboratory & Technical Innovation (LSAIT), Eldorado do Sul, Rio Grande do Sul, Brazil
***Corresponding author: benitobrito@gmail.com**

Avian pathogenic *Escherichia coli* (APEC) cause extra intestinal disease in chickens, turkeys, and other avian species via respiratory tract infection, giving rise to typical signs related to colibacillosis, such as: septicaemia, enteritis, granulomas, omphalitis, sinusitis, airsacculitis, arthritis/synovitis, peritonitis, pericarditis, perihepatitis, cellulitis, and swollen head syndrome. Disease caused by APEC strains results in significant financial losses to the poultry industry worldwide. This review provides essential information on the diagnosis of APEC. The information cited in this review will elucidate the various forms of APEC diagnosis while demonstrating the, lack of a precise diagnosis of this strain. The diagnoses mentioned herein reinforce the necessity to make progress toward a more accurate diagnosis of APEC to contribute to its reduction in poultry flocks.

Keywords: Escherichia coli; APEC; diagnosis; methods

Introduction

Substantial expansion in production within the poultry industry, for example in Brazil, predisposes it to a higher risk of infection by several microorganisms that may have an impact on the final quality of the chicken. Among these microorganisms, *Escherichia coli* (*E. coli*) is of great concern due to the economic losses its infection generates, with total or partial condemnation of carcasses, as well as the high cost of antibiotic treatment (Kabir, 2010).

E. coli belongs to the *Enterobacteriacea* family and is a rod-shaped, Gram-negative, non-sporulated, facultative anaerobic bacterium. It ferments lactose with the production of acid and gas when incubated at 44°C (Nakazato *et al.*, 2009). The bacteria approximately 1.1-1.5 µm in diameter and 1.0-6.0 µm in length, is normally motile due to the presence of flagella, usually fimbriated (Ewing, 1986), and colonises the

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gastrointestinal tract of both humans and birds. Diarrhoeal *E. coli* (DEC) are responsible for intestinal infections, and there are six described pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). The *E. coli* pathotype implicated in extraintestinal infections is ExPEC and may result in urinary tract infections (UTIs) by uropathogenic *E. coli* (UPEC) and sepsis/newborn meningitis associated *E. coli* (NMEC) (Kaper *et al.*, 2004).

Avian Pathogenic *Escherichia coli* (APEC); a pathotype of ExPEC, causes septicaemia characterised by air sacculitis, pericarditis, perihepatitis (Gross, 1994), swollen-head syndrome, salpingitis, omphalitis (Barnes *et al.*, 2008), cellulitis (Brito *et al.*, 2003), and colibacillosis in broiler chickens and laying hens (Barnes *et al.*, 2008). The infection is generally secondary to the environmental factors such as high rates of ammonia, moisture, dust, or infectious agents like viral and mycoplasmal infections and stresses (Gomis *et al.*, 2001; Barnes *et al.*, 2008; Dziva and Stevens, 2008).

The diagnosis of *E. coli* is based on clinical features and macroscopic lesions (Kabir, 2010), followed by inoculation of the agent in embryos or in 1-day-old chicks. Attempts to isolate the agent usually demand specific media like MacConkey (MAC) and eosin-methylene blue agar (EMB). Biochemical tests (*e.g.*, indol production, fermentation of glucose with gas production, presence of β-galactosidase, absence of hydrogen sulphite production, urease, and the inability to use citrate as a carbon source) are necessary to identify the isolated colonies (Dho-Moulin and Fairbrother, 1999). Strains of *E. coli* such as the DEC or ExPEC pathotype tend to share common characteristics. These characteristics are defined by O (lipopolysaccharide antigen, LPS), H (flagellar antigens), and K (capsular antigen). Antigens that are only O designate serogroups, and antigens that characterise serotype are called O:K:H (Kaper *et al.*, 2004). Another serological method includes enzyme-linked immunosorbent assay (ELISA) tests, most which are based on sonicating *E. coli* against two main APEC serotypes, O78:K80 and O2:K1 (Leitner *et al.*, 1990). Bell *et al.* (2002) developed an ELISA-based on fimbrial antigen. Molecular techniques such as PCR and real-time PCR are being developed for the detection of genes that are specific to the most important antigens: O and H. Other tools that are being used to characterise the isolates include ribotyping, sequencing, phylogeny, Multilocus Sequence Typing (MLST), and analysis of the virulence factors (VFs).

There are many diagnoses of APEC, but no centralised or detailed information about APEC yet exists. So the main purpose of this review article is to focus on the various aspects of the different diagnoses of APEC.

Pathogenicity test in day-old chicks

The pathogenicity test is the gold standard to confirm whether one *E. coli* strain is virulent. The subcutaneous inoculation method of APEC in embryos or 1-day-old chicks determines the virulence of these strains according to a 50% lethal dose. APEC isolates, when inoculated in these models, are able to kill them (Dho-Moulin and Lafont, 1982).

In addition, two- to four-week-old birds may be used to test the pathogenicity of the APEC strain. This model allows the reproduction of the disease as it occurs in the field. In this model, the sample is inoculated into the naso-pharynx (Smith *et al.*, 1985) or in the trachea (Goren, 1978) after a preliminary challenge with another agent because *E. coli* is usually secondary to infection by viral agents (infectious bronchitis virus, Newcastle disease), mycoplasma, or environmental conditions. Classic clinical signs of colibacillosis

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are weight loss, presence of fibrin in the air sacs, pericarditis and perihepatitis, contamination of the internal organs, and the presence of haemorrhage in the internal organs. This model confirms the pathogenicity of the inoculated strain (Dho-Moulin and Fairbrother, 1999).

Strains of *E. coli* inoculated directly into the air sacs are considered a high-performance model because in this situation, the preliminary action of triggering agents is not necessary. Inoculation in the air sacs allows more homogeneous lesions typical of colibacillosis compared with inoculations in the upper respiratory tract (Piercy and West, 1976).

Isolation and biochemistry

To cultivate the majority of microorganisms *in vitro*, culture media are required. Some of these media besides allowing the growth of microorganisms is used to differentiate one sample from another based on biochemical characteristics. In addition, selective culture media enable the growth of some pathogens and inhibit others because the media are used to differentiate closely related organisms.

Eosin methylene blue (EMB) agar is a differential medium used for the detection and isolation of Gram-negative intestinal pathogens initially described by Holt-Harris and Teague in 1916 and modified by Levine (1918). The dyes, eosin Y and methylene blue, are used as indicators of differentiation in response to fermentation of lactose and or saccharose in organisms that ferment lactose and those that do not. In addition, methylene blue acts as an inhibitor of Gram-positive organisms. Some enterobacteria or coliform groups may ferment saccharose more readily than lactose (Macfadden, 1985). Thus, the majority of EMB agar includes saccharose in their composition. Colonies of *E. coli* usually present a dark centre and a greenish metallic sheen due to the rapid fermentation of lactose whereas other organisms such as *Salmonella*, which do not ferment lactose or saccharose, produce colonies that are non-coloured or have a transparent amber colour.

MacConkey (MAC)'s agar is a selective medium that inhibits the growth of Gram-positive bacteria, especially enterococci and staphylococci, due to the presence of crystal violet and bile salts. MAC provides a better growth of Gram-negative bacteria. MACs are often used to detect and isolate all types of dysentery, typhoid, and paratyphoid organisms and differentiate *Salmonella thyposa* from coliform groups (Mossel *et al.*, 1962).

Bacteria grown on MAC are designated 'lactose fermenters'; the colonies are brick-red in colour and are surrounded by a zone of precipitated bile. The fermentation of lactose produces an acidic product, and these reactions provide the colour to the colonies. If the colonies grown on MAC are pink, the bacteria are Gram-lactose-fermenting. This usually occurs with the members of the *Enterobacteriaceae* family, including *Escherichia, Klebsiella, Enterobacter, Hafnia*, and *Citrobacter spp.*. Bacteria that are non-lactose-fermenting and do not belong to the coliform group are also able to grow on MAC, but the colonies are non-coloured and transparent and include members of *Enterboacteriaceae*, such as the genera *Proteus, Morganella, Providencia, Edwardsiella, Salmonella, Shigella* and *Yersinia*. The presence of crystal violet and bile salts in this medium avoids the grown of Gram-positive bacteria (Mossel *et al.*, 1962).

According to Park *et al.* (2010), the isolation of *E. coli* in Sorbitol MacConkey medium (SMAC) and SMAC plus cefixime and tellurite (CT-SMAC) were extensively used. These media may provide false positive results from sorbitol-nonfermenting bacteria

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such as *Hafnia alvei*, *Proteus* spp., *Providencia* spp., *Aeromonas* spp., and *Morganella morganii* (Fujisawa *et al.*, 2000; 2002). Furthermore, the recovery of injured *E. coli* O157:H7 cells in these media were poorly performed (Ray and Adams, 1984; Conner and Hall, 1994; McCarthy *et al.*, 1998; Taormina *et al.*, 1998). To avoid false positive results an improved medium, a modified version of SMAC with gentiobiose (GMAC) was tested and better supported the recovery of injured *E. coli* O157:H7. This medium does not contain antibiotics and has a low bile salt concentration, and these modifications allow a better recovery of injured cells than in CT-SMAC or SMAC (Park *et al.*, 2010).

E. coli responds positively to changes in the environment due to the enzymes that it possesses. The mechanisms that perform these changes have been identified: modulation, selective covalent modification, and inactivation. Thus, *E. coli* will inhibit or activate enzymes that promote their survival, either in the host or in the medium (Bettelheim, 1994).

The main biochemical characteristic of *E. coli* is the production of acid and gas after fermentation of glucose, maltose, mannose, mannitol, xylose, glycerol, rhamnose, arabinose, and sorbitol. The use of other sugars such as adonitol, saccharose, salicin, raffinose, ornithine, arginine, and dulcitol is variable. Positive responses are observed in motility, lysine, and indole production. Negative results are expected in tests like oxidase, citrate utilisation, urea hydrolysis, gelatin liquefaction, and H_2S (hydrogen sulphide) production (Bettelheim, 1994; Barnes *et al.*, 2008). Other tests like methyl red and Voges Proskauer are expected to be positive and negative, respectively (Barnes *et al.*, 2008).

A basic characteristic of *E. coli* is the ability to ferment glucose with the production of acid and gas. The conversion of lactose to glucose and galactose takes place due to the presence of the enzyme β-galactose. This enzyme is used for the differentiation of *E. coli* from *Salmonella sp*. and *Shigella sp*. (Bettelheim, 1994).

Serotyping

APEC strains are classified in serogroups as O:K:H serotypes. Serogroup O is related to the somatic antigen (Mellata *et al.*, 2012); capsular antigen refers to K1 (Barnes *et al.*, 2008); serogroup H encompasses flagella antigen; and the antigens type 1 (F1A), P (F11), as well as the curli fimbriae are concerned with fimbriae (Babai *et al.*, 2000; La Ragione *et al.*, 2002). According to Yerushalmi *et al.* (1990), there are 177 'O' antigens, 100 'K' antigens, and 56 'H' flagellar antigens. However, Lior (1994) cited 167 'O' antigens, 74 'K' antigens, 53 'H' antigens and 17 fimbriae antigens.

The somatic 'O' antigen, composed basically by polysaccharides, is characterised as an endotoxin released after lysis of *E. coli* (Perepelov *et al.*, 2007). O serogroups that are implicated in avian colibacillosis are O1, O2, O3, O4, O6, O8, O11, O15, O18, O21, O35, O36, O50, O64, O71, O74, O75, O78, O87, O88, O95, O100, O103, O109, O115, O119, O132, O141, and O152 (Gross, 1994; Dho-Moulin and Fairbrother, 1999; Mellata *et al.*, 2012). The most common serogroups in APEC are O1, O2, O8, O35, and O78 (Mellata *et al.*, 2012).

The presence of a fimbrial antigen, or antigen 'F', is related to the adhesion of *E. coli* to the cell surface. The fimbriae, also called *pili*, are sensitive or resistant to agglutination mannoses and are related to the presence of this carbohydrate in the cell (Barnes *et al.*, 2008). The majority of APEC possess type 1 fimbriae (Orskov and Orskov, 1992; La Ragione *et al.*, 2000a; 2002) and curli fimbriae. The curli, type 1, and P fimbriae APEC may possess avian *E. coli* 1 fimbriae (AC/1 fimbriae), a new fimbrial cluster of the S-fimbrial adhesin family that adheres to avian tracheal cells (Babai *et al.*, 2000); F1C,

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which adhere to buccal epithelial cells (Schmitz *et al.*, 1986); Dr fimbriae, which persist in kidney tissue (Ewers *et al.*, 2007); and type-1-like fimbriae (Van Den Bosch *et al.*, 1993; Pourbakhsh *et al.*, 1997).

Another antigen, the flagellin, designated as antigen 'H', is a protein that encodes the scourge of *E. coli* (Barnes *et al.*, 2008). The flagellar antigen can be found in most APEC, and the presence of this antigen characterizes the motility of APEC. The penetration of intestinal mucus, colonisation, invasion, and persistence in a SPF chick model is provided by flagella (La Ragione *et al.*, 2000a; 2000b). This antigen is not related to the virulence of *E. coli* (Barnes *et al.*, 2008).

The K capsular antigen is composed of polysaccharides (polysialic acid) and is found in the predominant APEC serotypes. It is associated with virulence. This antigen has been associated with extra-intestinal infections (Bliss *et al.*, 1996). Bree *et al.* (1989) identified the K antigen as a virulence determinant in avian O2:K1 isolates. Barnes (1994) found that the serogroups O1:K1, O2:K1, and O78:K80 are commonly involved in colibacillosis. The serotype O86:K61, to date, has not been isolated from commercial poultry; it is only associated with diarrhoeal disease in calves (Blanco *et al.*, 1993), pigs (Alexa *et al.*, 1995), and horses and implicated in cellulitis in broiler chickens on one occasion (Peighambari *et al.*, 1995). According to Moulin-Schouleur *et al.* (2007), the APEC serotypes O1:K1, O2:K1, and O18:K1 are potential zoonotic strains and belong to the same clonal group of human *E. coli* isolated from cases of neonatal meningitis, urinary tract infections, and septicaemia. The serogroup O25b:H4-ST131 (sequencing type 131), a UPEC strain, circulates between humans and animals; this strain has recently been detected in retail chicken (Vincent *et al.*, 2010).

Enzyme linked immunosorbent assay (ELISA)

ELISA is extensively used as a diagnostic tool in veterinary medicine as a quality control measure to screen food products for the presence of specific pathogens, such as *E. coli*; it is also used as an official veterinary diagnostic test for the detection of specific antigens or antibodies in production animals (*e.g.*, classical swine fever, Aujeszky disease, avian influenza, Newcastle disease) and for research use.

The ELISA principle is the binding of an antigen to its specific antibody. The test is performed on blood or urine and is used for measuring the amount of a specific protein or substance secreted or released from cells, such as infectious agents, allergens, hormones, or drugs (Gan and Patel, 2013). The technique is based on a five-step procedure: coat the microtitre plate wells with the antigen/antibody; block all unbound sites with milk to prevent false positive results; add primary antibody in the wells; add secondary antibody linked to an enzyme (*e.g.*, horseradish peroxidase, avidin, or biotin); and the substrate reacts with the enzyme to produce a coloured product, thus indicating a positive reaction. Many types of ELISA exist today, such as indirect, direct, sandwich, competitive (Gan and Patel, 2013), and a new type, multiple and portable ELISA (Balsam *et al.*, 2013).

Many commercial ELISA kits can identify *E. coli*. Some of them detect it in food products: SafePath® *E. coli* O:157 Microwell ELISA®, REAGEN™ *E. coli* O157:H7 Elisa Test Kit, and 3M™ TECRA™ *E. coli* 0:157. These tests are sandwich ELISAs utilising specific anti-*E. coli* O:157 antibodies. Abnova® laboratories possess a double antibody (sandwich) ELISA using an anti-*E. coli* O:157 antibody; the BIO K 345 *E. coli* F5 ELISA Kit® from Bio-X Diagnostics® detects the antibodies of the F5 (K99) pilus antigen of *E. coli*; and DAI® *E. coli* O:157 captures the antigen from stool samples. The *E. coli* protein (*E. coli* P) ELISA Kit® from MyBiosource® is a sandwich enzyme

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immunoassay technique to determinate the *E. coli* P in concentrations in serum, plasma, and tissue homogenates.

Studies from Van Den Bosch *et al.* (1993) and Pourbakhsh *et al.* (1997) demonstrated that *E. coli* isolates from chickens, expressed P fimbrial adhesins of serotype F11, as detected by ELISA, provide evidence that these fimbriae are produced *in vivo*. Dhama *et al.* (2013) reported that the ELISA test is more sensitive than an indirect haemagglutination test, which is a common method for detecting antibodies against *E. coli*. Two important pathogenic serotypes of sonicated *E. coli*, O78:K80 and O2:K1, are utilised to detect antibodies against APEC strains by ELISA, and another is based on fimbrial antigen (Lymberopoulos *et al.*, 2006).

Molecular biology

The expansion of molecular biology tools to evaluate the genetic variability of many bacterial strains such as *E. coli* (Versalovic *et al.*, 1991) occurred due to the discovery that prokaryotic genomes contain repeated sequences: repetitive extragenic palindrome (REP), or palindromic unit (PU) sequence (Gilson *et al.*, 1984), and the enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton *et al.*, 1991). The use of specific primers homologous to these regions after PCR reaction creates a pattern of amplified bands that is specific to each strain (Versalovic *et al.*, 1991).

To determine the phylogenetic groups of *E. coli* strains, Clermont *et al.* (2000) described a quick technique based on triplex PCR based on the detection of the *chuA* and *yjaA* genes and an anonymous DNA fragment designated as TSPE4.C2. This work evaluated 230 strains that had been previously grouped by other reference methods.

Carson *et al.* (2001) differentiated a total of 287 faecal *E. coli* from water resources by ribotyping to distinguish pooled faecal *E. coli* isolates from cattle, swine, horses, dogs, chickens, turkeys, and geese from that of human origin. Carson *et al.* (2001)'s results were accurate to discriminate between riboprint patterns of human and nonhuman origin.

Many molecular typing methods have been employed to study APEC, but none have revealed a specific genotype (Dziva and Stevens, 2008). Johnson *et al.* (2008) described a multiplex PCR panel targeting the five genes carried by plasmids considering that these genes are associated with highly pathogenic APEC strains: *iutA*, *hlyF*, *iss*, *iroN*, and *ompT*. This multiplex PCR that amplifies these five genes was used in 994 avian *E. coli* samples. The PCR distinguished APEC strains from the avian faecal *E. coli* isolates due to the presence of these five genes.

Schouler *et al.* (2012) identified four groups of virulence genes associated with APEC that interacted with the virulence genotyping of a greater collection from Europe with serotyping and experimental pathogenicity tests in 1-day-old chicks. The results obtained by Schouler *et al.* (2012) allowed the identification of more APEC strains than when using the serotyping method. These two approaches involving virulence genes proposed by Johnson *et al.* (2008) and Schouler *et al.* (2012) constitute a safe approach to identify strains of APEC in avian flock.

Other work involving virulence genes was proposed by Jeong *et al.* (2012). Virulence genes of 101 samples of APEC from Korea were characterised in order to examine the genetic background of this pathogen. The genes tested by Jeong included *fimC, tsh, hlyF, iroN, iucD, fyuA, irp2, iss, ompT, vat, lt, st, stx1,* and *stx2*. In Southern Brazil, Barbieri *et al.* (2013) genotyped 144 cellulitis isolates from broiler chickens and tested them for virulence in 1-day-old chicks and also characterised the isolates in terms of their resistance to 15 antimicrobial agents, their *E. coli* reference collection (ECOR) phylogenetic typing, and their clonal relationships.

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Stacy *et al.* (2014) recently described the prevalence of a newly discovered *E. coli* common pilus (ECP) for the subunit protein of the ECP pilus (ecpA) and ECP expression among APEC strains as well as the role of ECP in virulence. A PCR-based ecpA survey of a collection of 167 APEC strains has shown that 76% (127/167) were ecpA+. The data described in this research show that ecpA is highly prevalent among APEC isolates, its expression could be differentially regulated in these strains, and ECP plays a role in the virulence of APEC.

Another tool that may be useful to provide information about the APEC strains is the phylogenetic tree. According to Rojas *et al.* (2014), who performed a phylogenetic analysis based on multilocus sequence typing (MLST) of seven housekeeping genes, the phylogenetic tree assembled by them was able to cluster the different pathotypes together. An *in silico* virulence gene profile was also determined for each of these strains, according to the presence or lack of 83 well-known virulence genes described in pathogenic *E. coli* strains. APEC strains from Brazil and the United States and human strains from UPEC and DEC had genetic similarities, according to the MLST phylogeny and virulence profiles. These results show that APEC strains from poultry may be hazardous to humans.

Conclusions

Many diagnostic tests are available to identify strains of *E. coli*, but most of these tests do not solidly determine whether the strain is pathogenic. The gold standard to confirm the pathogenicity of *E. coli* strains is the inoculation of day-old chicks or embryos. Attempts to isolate the agent in selective media followed by analysis of biochemical characteristics provide information about the isolated agent, *i.e.*, this test will confirm if the agent is *E. coli* or *Shigella*, but it does not identify which strain. Thus, more accurate tests are needed. The O:H:K serotyping method, used by many laboratories, demonstrates the epidemiology of the strain to be identified, but does not demonstrate the pathogenicity of the sample.

Serological tests such as ELISA are well developed for analysis of samples from food and water. These tests use standard samples, based on fimbrial or somatic antigens, but this is not enough to assess whether the sample is pathogenic. The majority of ELISA tests on APEC samples are for research purposes only. For APEC strains, there is a lack of commercial ELISA tests available, and more studies using this kind of diagnostic test will help to identify APEC strains.

In molecular biology, many advances have been made that allow analysis of several virulence genes. But many such genes are not expressed normally - they are expressed only when the agent is in contact with the host. Hence, PCR is an important screening technique to detect genes that are related to the APEC strains virulence. However, the pathogenicity of the strain could be confirmed via inoculation in 1-old-day chick models displaying signs compatible with lesions of avian colibacillosis followed by death.

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