Multiplex PCR is the first technique to allow the specific and sensitive detection of avian pathogenic *Escherichia coli* (APEC)

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Introduction

Infections with avian pathogenic *E. coli* (APEC) cause colibacillosis, an acute disease of poultry which comprises both local and systemic infections and manifests itself in a range of different organ lesions. The principal target sites are the respiratory tract (lung, air sacs), which acts as a port of entry for APEC, and at a later stage also the serosa of the internal organs, the peritoneum, oviduct, meninges and joints. The inflammatory lesions in these organs usually result in acute death. Colibacillosis causes heavy economic losses worldwide (BARNES and GROSS, 1999). In addition to APEC, environmental conditions (poor housing, contaminated feed/drinking water, overcrowding) and host susceptibility (immunosuppression, stress, injuries etc.) are also implicated in outbreaks of the disease (DHO-MOULIN and FAIRBROTHER, 1999). This was discussed in a previous paper by PHILIPP and VOSS in the 2/2001 issue of LOHMANN INFORMATION (PHILIPP and VOSS, 2001).

Most avian *E. coli* isolates are normal inhabitants of the intestinal microflora of poultry and are therefore commonly present in the birds' environment. These isolates perform important metabolic functions for the host and are apathogenic. But by exchanging genetic material these apathogenic bacteria can become pathogenic if the newly acquired genetic material codes for specific virulence factors. Such isolates are then capable of causing infections of the colibacillosis disease syndrome. The causal agents most commonly isolated are strains of the serovars O1:K1, O2:K1 and O78:K80, which are identified in up to 61 % of tested isolates (DHO-MOULIN and FAIRBROTHER, 1999; PHILIPP and VOSS, 2001). But it also implies that at least 40 % of isolates remain quite simply undetected with current diagnostic techniques, which still rely on serotyping, because these strains are not covered by the aforementioned serovars. Yet serotyping remains one of the most widely used diagnostic tools for identifying avian *E. coli* wild-type strains as APEC strains. This lack of diagnostic power as a result of using agglutinating sera constitutes an unacceptable diagnostic gap.

Genotypical methods for the detection of specific virulence factors of APEC are a more effective way of reliably identifying avian pathogenic *E. coli*. Definitive identification of APEC is the key to gaining further insights into the significance of virulence factors in the pathogenesis of colibacillosis. Our knowledge of the exact bacterial characteristics that play a role in this disease process is still relatively poor, but in recent years infection experiments have shed some light on the relationship between the various factors and virulence. These virulence factors facilitate adhesion of avian pathogenic *E. coli* to the epithelium of the respiratory tract, their multiplication and spread within the host, the development of resistance to host defence mechanisms, manifestations in organs and the induction of cytopathic effects. Table 1 below gives an overview of potential virulence factors of APEC isolates identified to date, which can in principle be used as target genes for the determination of APEC.

Fimbria are fibrous structures that can be seen on the surface of numerous bacteria with the aid of an electron microscope. F1 fimbria, P fimbria and curli are believed to be involved in the pathogenesis of colibacillosis. F1 fimbria are expressed by many enterobacteria, both pathogenic and apathogenic, although several studies have shown that avian pathogenic *E. coli* express them far more frequently than do apathogenic isolates (DOZOIS et al., 1992; WOOLEY et al., 1992). F1 fimbria enable APEC isolates to adhere to the epithelial cells of the respiratory tract (trachea, lung and air sacs). They also seem capable of protecting *E. coli* strains from bactericidal activities of the host (ORNDORFF, 1994). P fimbria are often found in *E. coli* isolates that cause extraintestinal infections, especially of the upper urinary tract in humans and dogs and septicaemias in pigs (JOHNSON et al., 2000).

Table 1: Virulence factors of avian pathogenic *E. coli*

Virulence factor	Pathogenic significance
Fimbria	
➢ F1 (type1) fimbria	- Adhesion to epithelial cells of the respiratory tract
➢ P fimbria	- Adhesion to internal organs
➢ Curli	- Protection against phagocytosis
Iron acquisition systems	
➢ Aerobactin	- Removal of iron from the host
➢ Yersinabactin	- Growth and rapid multiplication
Haemolysins	**tsh only**
➢ Haemolysin E	- Development of lesions and fibrin deposits in the air sac
➢ Temperature-sensitive haemagglutinin (tsh)	- Destruction of erythrocytes
Anti-host defence systems	
➢ External membrane proteins ➢ Iss protein ➢ Lipopolysaccharide complex ➢ K (1) capsule ➢ ColicinV production	- Protection against bactericidal mechanisms of the host in the blood stream, mainly by inhibition of the complement fixation system
Toxins and cytotoxins	
➢ Heat-stable toxin (EAST-1) ➢ Shiga toxin (Stx2f) ➢ Cytotoxin (verotoxin) ➢ Flagellar toxin	- Still poorly understood, probably interfere with metabolic processes in the cell - Vacuolisation of cells

It has been proved by infection experiments that these fimbria are also expressed by avian pathogenic *E. coli* isolated from the lungs, air sacs and internal organs but not from the trachea (DOZOIS et al, 1994; van den BOSCH et al., 1993; VIDOTTO et al., 1997). Moreover, they seem capable of protecting APEC isolates against phagocytosis (POURBAKHSH et al., 1997 and 1997a). The role of curli fibres in the pathogenesis of the disease is not yet fully understood. It is supposed that they facilitate bacterial adhesion and colonisation, especially in the early stage of the infection (MAURER et al., 1998; OLSEN et al., 1998).

The siderophores aerobactin and yersiniabactin enable microorganisms to deprive the host of essential iron, which the bacteria need for growth and multiplication. Without siderophores bacteria would be unable to survive in the host because concentrations of freely available iron in body fluids of animals and humans are very low (CHIPPERFIELD et al., 2000). Numerous studies have demonstrated that aerobactin is an APEC-associated virulence factor. 70 to 80 % of *E. coli* isolated from colibacillosis possess these siderophores (DOZOIS et al., 1992; NGELEKA et al., 1996; JANSSEN et al., 2001). The second iron acquisition system found in APEC isolates was yersiniabactin (GOPHNA et al., 2001; JANSSEN et al., 2001). This siderophore was originally described in Yersinia bacteria.

Some APEC also possess haemolysins, which are capable of damaging erythrocytes. This rare APEC phenotype can be demonstrated on blood agar plates. A completely new haemolysis gene (hlyE) was identified in an avian pathogenic *E. coli* isolate (REINGOLD et al., 1999), but numerous other studies failed to detect it by genotypical methods in APEC isolates, nor was haemolysis of the tested isolates observed with phenotypical techniques (GOMIS et al., 2000; KNÖBL et al., 2001). Temperature-sensitive haemagglutinin (tsh) on the other hand seems to play a significant role in the pathogenesis of colibacillosis, firstly because it has been detected in a large proportion of APEC isolates, while *E. coli* isolates from clinically healty animals possess no tsh (MAURER et al., 1998), and secondly because it is closely associated with lethality in chickens (DOZOIS et al., 2000). With the aid of this temperature-sensitive haemagglutinin APEC seem able to induce lesions and fibrin deposits in the air sacs. The exact role of tsh in the pathogenesis of colibacillosis has not yet been fully established, however.

The ability of APEC to escape certain host defence systems, for example bactericidal activity in the serum, is indispensable for a successful pathogenesis. This ability is due to a capsule, colicin, lipopolysaccharides (LPS), external membrane proteins and increased serum survival protein (iss). The last-named iss protein in particular is closely associated with APEC isolates (PFAFF-McDONOUGH et al., 2000). Like most of the virulence factors mentioned earlier, it induces resistance to the complement fixation system of *E. coli* isolates, thereby considerably enhancing their virulence. The exact mechanism of action is again not fully understood.

Finally, the role played by the few toxins produced by APEC isolates in the symptomatology of colibacillosis is still largely unknown. They are frequently associated with certain clinical manifestations or certain poultry species. Cytotoxins for instance were found with above-average frequency in avian *E. coli* isolates isolated from birds with "swollen head syndrome" (SHS) (PARREIRA et al., 1998; SALVADORI et al., 2001). Shigatoxin stx2f on the other hand has so far only been demonstrated in pigeons (SCHMIDT et al., 2000). Other toxins that may be implicated in the pathogenesis of colibacillosis are the heat-stable toxin EAST-1 and flagellar toxin, which has been used as the basis for a vaccine.

Accurate identification of *E. coli* wild-type strains as APEC isolates is essential if flock-specific vaccines are to be produced for instance. As mentioned earlier, serytopying is not a suitable method for the reliable detection of APEC. Genotypical methods are preferable for this purpose, like DNA-DNA hybridisation or polymerase chain reaction (PCR) for example. The most suitable target genes for a specific and sensitive assay are currently the above-mentioned APEC virulence-associated genes because their use enables not only the specific detection of APEC but also the simultaneous prediction of the virulence potential of the bacteria. The Institute of Microbiology and Animal Epidemic Diseases in Berlin in collaboration with the veterinary laboratory of Lohmann Tierzucht GmbH has designed a multiplex PCR diagnostic assay which is intended to replace serotyping as the most widely used diagnostic technique in the future. This multiplex PCR, described here for the first time, makes it possible to differentiate between APEC and apathogenic *E. coli* within a few hours. It can detect six virulence-associated genes (astA, irp2, iss, papC, iucD and tsh) simultaneously. The genotypical co-detection of several virulence-associated genes and the simple use of bacterial colonies of the *E. coli* wild-type strain being tested makes it a rapid, cost-efficient and accurate diagnostic tool.

Own investigations

Numerous *E. coli* wild-type strains have been serotyped and additionally genotyped by DNA-DNA hybridisation and polymerase chain reaction since 1999 at the Institute of Microbiology and Animal Epidemic Diseases in Berlin as part of a doctoral thesis. All isolates are from poultry that had died from colibacillosis and were sent in by the veterinary laboratory of Lohmann Tierzucht GmbH. Among the objectives of the research was to establish a diagnostic technique that is capable of providing rapid and reliable predictions about the pathogenicity of these isolates.

Materials and methods

Isolates

A total of 150 *E. coli* isolates from chickens of various ages that had died from colibacillosis were tested. The majority of isolates came from laying hens, others from broilers, broiler breeders and turkeys. These 150 *E. coli* field strains had been isolated over an 11-year period from various poultry operations in Germany, but also in Egypt, the UK and Jordan. One to six isolates usually came from the same farm. The *E. coli* strains were cultured on Gassner and blood agar plates at 37 °C.

DNA-DNA hybridisation

DNA-DNA hybridisation allows the detection of identical or very similar sequences of the nucleic acid being tested and of a specific marker probe. The 150 avian pathogenic *E. coli* under investigation were tested for the following ten virulence-associated genes: fimC, papC, tsh, hlyE, iucD, fyuA, irp2, iss, astA and stx2f. The respective DNA probes were produced with the "PCR DIG Probe Synthesis Kit" (Roche Diagnostics GmbH, Mannheim). The hybridisation procedure and the visualisation were carried out in accordance with the instructions for the "DIG Luminescent Detection Kit" (Boehringer Mannheim GmbH, Mannheim).

Polymerase chain reaction (PCR)

For verification purposes the *E. coli* field isolates were additionally tested by polymerase chain reaction (PCR) for the aforementioned virulence-associated genes. PCR enables the exponential amplification of a specific DNA range by means of two oligonucleotide primers and Taq-DNA polymerase. All oligonucleotide primers were synthesised by MWG Biotech, Ebersberg. Chromosomal DNA isolated by heat lysis was used as template DNA.

Multiplex PCR

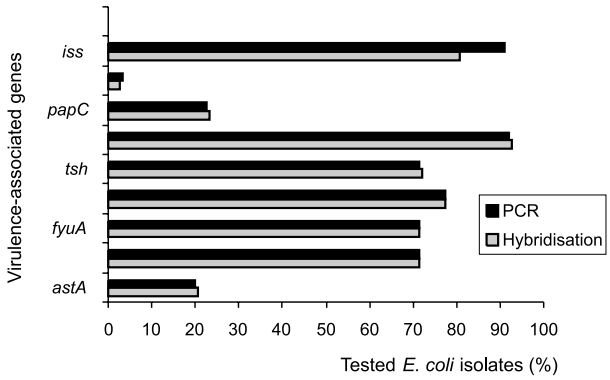
In order to detect several virulence-associated genes relevant for colibacillosis a multiplex PCR technique was designed. This allows the simultaneous identification of two or more DNA fragments in a single PCR assay. The following primer pairs were used for the detection of the corresponding genes in the multiplex PCR: EAST, iss, HMWP-2, papC, AERA and tsh. The chromosomal DNA processed by heat lysis and for comparison a colony of the isolate to be tested, taken directly from the LB agar plate incubated overnight, were used as template DNA.

Results

The prerequisite for the establishment of a multiplex PCR was the prior testing of the APEC wild-type strains by DNA-DNA hybridisation and polymerase chain reaction. The purpose of these assays was to determine the frequency with which the various virulence-associated factors occur in the resident APEC population. These data would then be used for identifying those genes that are suitable as target genes for a multiplex PCR.

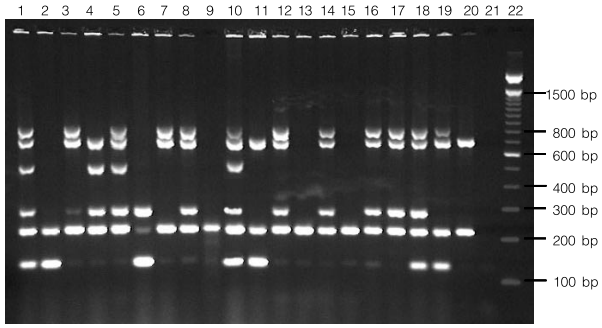
The aforementioned virulence-associated genes were detected fairly consistently with these assay methods. Only the fimbrial genes fimC and papC, the tsh gene coding for the temperature-sensitive haemagglutinin, and the toxin gene astA were each detected more often in one isolate by DNA-DNA hybridisation than by PCR. In two isolates different results were obtained with the various methods as regards the detection of the aerobactin-coding iucD. The yersiniabactin genes fyuA and irp2, essential for iron acquisition, were found in identical numbers in the 150 tested *E. coli* field isolates. Random tests for the presence of the iss gene coding the corresponding increased serum survival protein, which is important for serum resistance, the haemolysin gene hlyE and the toxin gene stx2f were performed by PCR in 22 (iss), 64 (hlyE) and 87 (stx2f) *E. coli* isolates respectively; the results of these tested isolates were in complete agreement with those of the DNA-DNA hybridisation. Figure 1 shows the distribution of the virulence-associated genes.

Figure 1: Detection of virulence-associated genes by DNA-DNA hybridisation and polymerase chain reaction (PCR)



The isolates were tested by multiplex PCR for six virulence-associated genes simultaneously. They are the toxin gene astA, the genes irp2 and iucD which are important for iron acquisition, the iss gene which plays a role in anti-host defence, the fimbrial gene papC and tsh, which codes the corresponding protein. Initially 2 µl heat lysis DNA and one colony from each strain to be tested were used as template DNA. The results of the multiplex PCR, obtained with colonies, were identical to those of the heat lysis so that eventually only colonies were used as template DNA in order to simplify the diagnostic procedure. Figure 2 shows an example of an electropherogram from a multiplex PCR assay in which 19 *E. coli* field isolates had been tested for the presence of the six virulence-associated genes.

Figure 2: Electropherogram of a multiplex polymerase-chain reaction (1.5 % agarose gel, 2.5 h, 90 V)



1: Positive control IMT 2540, 2: IMT 4537, 3: IMT 4539, 4: IMT 4641, 5: IMT 5124, 6: IMT 2111, 7: IMT 2488, 8: IMT 4532, 9: IMT 5127, 10: IMT 5215, 11: IMT 5125, 12: IMT 2297, 13: IMT 2275, 14: IMT 2293, 15: IMT 2282, 16: IMT 2283, 17: IMT 2271, 18: IMT 2272, 19: IMT 2264, 20: IMT 2265, 21: Black value, 22: Marker 100 bp-ladder
The fragment sizes of individual PCR amplificates are: *astA* 120 bp, *iss* 219 pb *irp2* 287bp, *papC* 519bp, *iucD* 711 und *tsh* 823bp.

Discussion

Colibacillosis is a disease of poultry which occurs worldwide and is associated with heavy economic losses. It presents both as a secondary infection (mainly after respiratory tract infections) and as a primary infection on farms with clinically inconspicuous flocks. Numerous in vitro and in vivo studies have been conducted to date in order to gain insights into the pathogenesis of colibacillosis, but the exact mechanisms by which the virulence factors of avian pathogenic *E. coli* cause infections are not yet fully understood. There is also a shortage of epidemiological studies which might provide more information about the spread and introduction of the disease into affected farms (TABLANTE et al., 1999; JANSSEN et al., 2001). This prompted us to genotype numerous *E. coli* wild-type strains by DNA-DNA hybridisation and polymerase chain reaction at our Institute. The intention was to gain more information about the incidence and distribution of the virulence-associated genes in APEC. The tests also formed the basis for the establishment of a diagnostic multiplex polymerase chain reaction assay (multiplex PCR).

References from the literature and comments by institutes engaged in diagnostic work confirm that the differentiation between avian pathogenic (APEC) and apathogenic *E. coli* isolates poses a major problem. The unsatisfactory serotyping technique remains the most widely used diagnostic tool for the identification of APEC isolates. The serovars O1:K1, O2:K1 and O78:K80 are most often isolated from the causal agents of colibacillosis, but only about half of the isolates can be detected with these three antisera (DHO-MOULIN and FAIRBROTHER, 1999). This provided the motivation for establishing a diagnostic multiplex PCR to replace serotyping as the most widely used

diagnostic technique in the future. The multiplex PCR enables the simultaneous detection of six virulence-associated genes (astA, irp2, iss, papC, iucD and tsh). As it eliminates processing of the genomic DNA, the multiplex PCR is a rapid, cost-efficient and accurate diagnostic tool.

The basis for the establishment of a multiplex PCR assay and its routine use were incidence and distribution of virulence-associated genes in avian pathogenic *E. coli* field isolates, determined by DNA-DNA hybridisation and PCR, and verification of the validity of the PCR. The results obtained with the two methods in detecting the genes in the isolates were almost identical and multiplex PCR can be recommended as a diagnostic technique. As the iron-acquiring genes irp2 and fyuA were always detected in combination, the multiplex PCR assay is consequently also capable of identifying the presence of fyuA. The virulence-associated genes stx2f and hlyE were detected only in a few isolates in this study and would therefore appear to play no significant role in the pathogenesis of colibacillosis.

The multiplex PCR assay designed in collaboration with the veterinary laboratory of Lohmann Tierzucht GmbH enables a reliable diagnosis to be performed. It is capable of detecting avian pathogenic *E. coli* isolates in clinical cases and of differentiating these clearly from apathogenic *E. coli*. Based on the distribution of the virulence-associated genes the procedure also facilitates the selection of vaccinal strains that have proved most pathogenic for poultry flocks. Finally, it is possible with multiplex PCR to identify sources of infection with a view to preventing the introduction of avian pathogenic *E. coli* isolates to other farms and the resulting rapid spread of colibacillosis.

Abstract

Serotyping is currently the most widely used diagnostic tool for identification of avian pathogenic *E. coli* isolates. This method has two major disadvantages, however. Firstly, it is unreliable since a large number of APEC wild-type strains do not belong to the usual serovars O1:K1, O2:K1 or O78:K80. Secondly, even a positive serotyping result does not predict the virulence potential of the isolate concerned. This led to the establishment of a multiplex PCR assay, based on genotypical analysis of numerous APEC strains by DNA-DNA hybridisation and polymerase chain reaction (PCR), to allow the rapid and cost-efficient characterisation of *E. coli* wild-type strains and their identification as avian pathogenic. The multiplex PCR technique is also capable of identifying the most highly pathogenic *E. coli* isolates in a flock. These isolates can be used as the basis for the production of a powerful vaccine to be used against APEC infections. By researching the chain of infection new and effective controls can be put in place to prevent the rapid spread of avian pathogenic *E. coli*. Finally, knowledge of the incidence and distribution of virulence-associated genes can be utilised for further epidemiological studies in order to gain new insights into the pathogenesis of avian colibacillosis.

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