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Diagnostic Strategy for Identifying Avian Pathogenic *Escherichia coli* Based on Four Patterns of Virulence Genes

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In order to improve the identification of avian pathogenic *Escherichia coli* (APEC) strains, an extensive characterization of 1,491 *E. coli* isolates was conducted, based on serotyping, virulence genotyping, and experimental pathogenicity for chickens. The isolates originated from lesions of avian colibacillosis ($n = 1,307$) or from the intestines of healthy animals ($n = 184$) from France, Spain, and Belgium. A subset (460 isolates) of this collection was defined according to their virulence for chicks. Six serogroups (O1, O2, O5, O8, O18, and O78) accounted for 56.5% of the APEC isolates and 22.5% of the nonpathogenic isolates. Thirteen virulence genes were more frequently present in APEC isolates than in nonpathogenic isolates but, individually, none of them could allow the identification of an isolate as an APEC strain. In order to take into account the diversity of APEC strains, a statistical analysis based on a tree-modeling method was therefore conducted on the sample of 460 pathogenic and nonpathogenic isolates. This resulted in the identification of four different associations of virulence genes that enables the identification of 70.2% of the pathogenic strains. Pathogenic strains were identified with an error margin of 4.3%. The reliability of the link between these four virulence patterns and pathogenicity for chickens was validated on a sample of 395 *E. coli* isolates from the collection. The genotyping method described here allowed the identification of more APEC isolates with greater reliability than the classical serotyping methods currently used in veterinary laboratories.

Colibacillosis is the major cause of morbidity and mortality in poultry and is responsible for significant economic losses worldwide. Avian pathogenic *Escherichia coli* (APEC) induces different syndromes in poultry, including systemic and localized infections, such as respiratory colibacillosis, acute colisepticemia, salpingitis, yolk sac infection, and swollen-head syndrome. The most common form of colibacillosis is characterized by an initial respiratory disease in 3- to 12-week-old broiler chickens and turkeys, which is usually followed by a systemic infection with characteristic fibrinous lesions (airsacculitis, perihepatitis, and pericarditis) and fatal septicemia. The infection is generally initiated or enhanced by predisposing agents, such as mycoplasmal or viral infections, and environmental factors (3, 18).

Two major issues currently make it difficult to control avian colibacillosis, namely, the lack of a reliable method to identify the causative strains of *E. coli* and the fact that currently available vaccines are not totally effective, despite the recent identification of protective vaccine antigens (42). These factors are due to the diverse characteristics of APEC strains preventing the identification of common properties, which could be used as a basis for diagnostic methods and vaccination.

Although APEC strains clearly belong to the phylogenetic group of extraintestinal pathogenic *E. coli*, numerous studies have shown wide serological diversity (7, 43). Predominant serogroups are O1, O2, and O78, but they account for only 15 to 60% of isolates depending on the study (18, 26, 36, 47).

Several virulence genes have been demonstrated to be implicated in avian colibacillosis, including those encoding for adhesins (F1, P, and Stg fimbriae, curli, and EA/I), anti-host defense factors (OmpA, Iss, lipopolysaccharide, and K1), iron acquisition systems (aerobactin, Iro proteins, yersiniabactin, and the Sit iron acquisition locus), autotransporters (Tsh, Vat, and AatA), the phosphate transport system, sugar metabolism, and the IbeA protein (1, 12, 13, 24, 35, 38, 48).

However, numerous studies have demonstrated that these virulence factors (VFs) are rarely all present in the same isolate, showing that APEC strains constitute a heterogeneous group. Different isolates may harbor different associations of virulence factors, each one able to induce avian colibacillosis.

This diversity currently hinders clear identification of an avian *E. coli* isolate as a pathogenic or nonpathogenic strain. However, there is a significant difference in gene prevalence between *E. coli* strains isolated from chickens with colibacillosis and those isolated from the feces of apparently healthy birds (47). Individually, virulence factors, such as adhesins, are not reliable tools for diagnosis because of their low incidence in avian pathogenic isolates (56).

Serotyping remains the most frequently used diagnostic method in laboratories, but it only allows the identification of a limited number of APEC strains. This method cannot therefore be used as an effective diagnostic tool, particularly since serotype does not reflect the virulence trait. Various studies have high-

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TABLE 1 Origins of *E. coli* isolates[a]

Isolate type and origin	No. of isolates			
	Chicken	Turkey	Duck	Total
L isolates				
France	293	330	153	776
Spain	427	19	1	447
Belgium	84	0	0	84
Total	804	349	154	1,307
I isolates				
France	94	50	0	144
Spain	39	0	0	39
Belgium	1	0	0	1
Total	134	50	0	184
Total	938	399	154	1,491

[a] *E. coli* isolates were collected from the internal organs or blood of animals showing typical symptoms and/or macroscopic lesions of avian colibacillosis (L isolates) or from the intestinal content of healthy animals without lesions at necropsy (I isolates).

lighted the possibility of using some virulence factors to identify APEC strains. They have attempted to determine a common identification scheme, allowing better identification of APEC strains than serotyping. These methods are mostly based on the detection of virulence factors carried on colicin V (ColV) plasmids using multiplex PCR. One study described a multiplex PCR protocol to detect the presence of *iss*, *tsh*, *iucC*, and *cvi* (55). Another described a minimum number of genes that could be used to identify an APEC strain, namely, *iutA*, *hlyF*, *iss*, *iroN*, and *ompT* (33). In a third study, a multiplex PCR protocol was designed to detect the presence of eight virulence genes (toxin genes *astA* and *vat*, the increased serum survival protein gene *iss*, iron acquisition genes *irp2* and *iucD*, adhesin genes *papC* and *tsh*, and the ColV plasmid operon genes *cva-cvi*) (27).

The present study involved extensive characterization of a large collection of European APEC isolates, based on serotyping, identification of virulence factors, and experimental pathogenicity for chickens. Statistical analysis, based on supervised learning methodology, allowed us to define several sets of virulence factors accounting for the majority of pathogenic strains and which could be used for diagnosis.

MATERIALS AND METHODS

Bacterial strains. A total of 1,491 *E. coli* isolates were collected over an 8-year period (1992 to 2000) in France ($n = 920$), Spain ($n = 486$), and Belgium ($n = 85$), from chickens ($n = 938$), turkeys ($n = 399$), and ducks ($n = 154$) (Table 1). Most isolates ($n = 1,307$) were from characteristic lesions of animals exhibiting clinical symptoms of different forms of colibacillosis (L strains), and 184 isolates were collected during the same period from the intestinal content of healthy animals with no characteristic symptom or lesion of colibacillosis at necropsy (I strains).

Isolates were collected by authorized diagnostic laboratories in each country and sent to a single laboratory. Each isolate was checked for purity and identified as *E. coli* using API 20E (bioMérieux, Inc.). Samples of each isolate were prepared from a single culture and used to inoculate conservation agar tubes (Bio-Rad) to be dispatched to the various collaborators. The same culture was also used to inoculate four tubes with brain heart infusion broth (Difco Laboratories) containing 20% glycerol, which were kept at −80°C.

Serogrouping. Determination of O antigens was carried out by agglutination according to the method described by Blanco et al. (5) and using all available O (O1 to O181) antisera. The antisera were obtained and adsorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O antisera were produced in the Laboratorio de Referencia de *Escherichia coli* (Lugo, Spain).

Virulence genotyping. The presence of genes encoding virulence factors was detected using PCR amplification. Crude DNA extracts were prepared by a rapid boiling method (51). A single PCR assay was used to detect *sfa-focDE* (operons encoding S fimbriae and F1C fimbriae), and nine multiplex PCR assays were performed to detect simultaneously: (i) *fimA* (with consensus primers), *fimA* variant ($fimAv_{MT78}$), and *fimH*; (ii) *neuC*, *felA*, and *papC*; (iii) $papG_{J96}$ class I, $papG_{IA2}$ class II, and $prsG_{J96}$ class III; (iv) *tsh* and *iutA*, (v) *cdt* (variants 1 to 4); (vi) *cnf1* and *cnf2*; (vii) VT1 and VT2 encoding genes; (viii) LT encoding gene; and (ix) STa and STb encoding genes. Strains were noted as F1 positive when both *fimA* (or $fimAv_{MT78}$ variant) and *fimH* genes were present and as P(F11) positive when the *felA*, *papC*, and a variant of *papG* genes were present. Gene *iutA* accounted for the aerobactin iron acquisition system and *neuC* for K1 antigen. The corresponding primers are listed in Table S1 in the supplemental material. DNA fragments were amplified in a 25-μl PCR mix containing 1 U of *Taq* DNA polymerase (Promega), 25 pmol of the forward and reverse primers, and 5 nmol of each deoxynucleoside triphosphate (Promega) in 1× buffer. The PCR conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, annealing for 1 min at 72°C for at least 30 s according to the size of the amplified fragment (1 min/kbp), and then a final extension at 72°C for 10 min. The *E. coli* strains used as positive controls in PCR assays were as follows: BEN 2908 (17, 52) for *fimA*, $fimAv_{MT78}$, *fimH*, *neuC*, and *iutA*; MT189 (19) for *felA* and *papC*; χ7122 (21) for *tsh*; KH576 (61) for *iutA*; 536 (29) for *sfa-focDE*; E6468/62 (53) for *cdt*; JJ48 ($papG_{J96}$), HB101/pDC1 ($papG_{IA2}$), and P678-54/pJFK102 ($PrsG_{J96}$) for *papG* class I, *ppaG* class II, and *papG* class III, respectively (31); MR199 and B26a for *cnf1* and *cnf2* (8); 933 for *vt1* and *vt2*; PD32b for the STa, STb, and VT2e encoding genes; and IP102a for the LT and STb encoding genes (9). The negative control strains were *E. coli* MG1655 (10) and the nonpathogenic *E. coli* avian strain EC79 (16).

Screening for the F17, *afa*, and *eae* encoding genes was performed using colony hybridization. The corresponding probes were obtained, purified, and labeled, and DNA colony hybridization was performed as previously described (56). The positive control strains were 25KH9 (F17+) (39), A30 (Afa+) (37), and E2348/69 (Eae+) (30), and the negative control strain was the HS strain (44). In addition, labeled cloned fragments of the APEC strain BEN2908 (*aec26* [A9], *sitA* [A12], *tkt1* [D1], frz_{orf4} [D7], and *aec4* [D10]) were prepared and hybridized with dot blots prepared from crude extracts of genomic DNA as previously described (52). The positive and negative control strains were BEN2908 and EC79, respectively.

Detection of cytotoxic activity of bacterial lysates and supernatants. Detection of toxins was also conducted using phenotypic assays. For detection of LT, VT1, VT2, CNF1, and CNF2, filtrates of cultures treated with mitomycin were inoculated into Vero and HeLa cells. For STa enterotoxin detection, extracellular fluids were assayed by the infant-mouse test, and sonic extracts were tested for necrosis using the rabbit skin test (6). Morphological changes of the HeLa cells characteristic of cytolethal distending toxin (CDT) were determined on nonconfluent HeLa cell monolayers in 96-well plates incubated with culture supernatants and with sonic lysates obtained from 48-h bacterial cultures as previously described (59). *E. coli* strain DH5α (51) was used as a negative control, and all experiments were conducted in triplicate.

Virulence for chicks. Groups of five 1-day-old specific-pathogen-free chicks were inoculated subcutaneously with 0.5 ml of an overnight culture in LB-Miller broth without agitation (the inoculum in stationary phase was $\sim 10^8$ CFU) as previously described (16), and the mortality was recorded at 4 days postinoculation. Strains were classified as pathogenic when at least one chick died (21). The nonpathogenic *E. coli* avian strain EC79 (serogroup O2) (16) was used as a control (no chicks died). The housing, husbandry, and slaughtering conditions conformed to European

Union guidelines for the care and use of laboratory animals. The animal experimentation authorization number for people directing these studies was 37-041.

Statistical analysis. The prevalence of virulence factors was compared using a chi-square test. *P* values of <0.05 were considered significant.

The association of virulence factors with pathogenicity was determined by a tree-based modeling method. A classification tree (11) was constructed using the R package rpart, version 3 (58). This method prepares a binary tree using a divisive algorithm from a top node (called the root) to terminal nodes (called the leaves). The top node contains all strains. At each step of the algorithm, a node is partitioned into two subnodes corresponding to the presence or absence of a virulence factor. The splitting factor is determined to obtain the purest subnodes according to pathogenicity.

RESULTS

Four associations of virulence genes are correlated with pathogenicity. A statistical analysis was performed to identify groups of virulence genes preferentially associated with APEC strains. This required accurately identifying *E. coli* isolates as pathogenic or nonpathogenic. Thus, among the collection of 1,491 strains, a sample of 460 isolates was tested for pathogenicity using the 1-day-old chick lethality test. Strains were classified as pathogenic (P; of five chicks inoculated, one to five died) or nonpathogenic (NP; of five chicks inoculated, none died).

Based on the lethality test, 352 isolates were identified as pathogenic (310 strains isolated from lesions of diseased birds [L isolates] and 42 strains isolated from the intestine of healthy animals [I isolates]) and 108 isolates (47 L isolates and 61 I isolates) as nonpathogenic. Among the pathogenic strains, 196 killed 5 chicks, and 41, 33, 38, and 44 killed 4, 3, 2, or 1 chick, respectively. Pathogenic I isolates did not differ from pathogenic L isolates when considering the number of dead chicks. It is not surprising to observe that some I isolates were virulent, since it is well established that the chicken intestine is the main reservoir of pathogenic strains (4, 25). Notably, 47 strains isolated from lesions were nonpathogenic. They were of various serogroups and scattered in the classification tree. This observation confirms the fact that isolation of an *E. coli* strain from a pathological lesion is not a sufficient criteria to classify it as a pathogen.

Among the 460 isolates, 84 serogroup O isolates were observed. Six serogroups accounted for 56.5% of pathogenic strains: O78 (17.5%), O2 (17.3%), O8 (2.0%), O18 (9.0%), O5 (4.5%), and O1 (6.0%) (Table 2). A total of 22.5% of nonpathogenic isolates belonged to these six serogroups (Table 2). These six most common serogroups were the same whatever the geographical origin (France, Spain, or Belgium) or animal species (chicken, turkey, or duck) (data not shown). Nontypeable isolates were less frequently observed among P strains (6.5%) than among NP strains (13.9%) (data not shown).

The presence of 19 associated virulence genes on the 460 isolates was detected using PCR assays and hybridization (see Table S2 in the supplemental material). The predominant virulence-associated genes on pathogenic strains were *sitA* (96.3%), F1 fimbriae encoding genes (86.4%), and *iutA* aerobactin gene (82.7%) (Table 2). Other genes were present in 20 to 60% of the pathogenic strains: *tsh*, frz_{orf4}, *tkt1*, *aec4*, P(F11) fimbriae encoding genes, *aec26*, and *neuC* (K1 antigen). Highly significant differences ($P <$ 1‰) were observed in the frequency of these virulence genes between P and NP isolates (Table 2). Five virulence genes were present in fewer than 10% of the P isolates: *f17*, *sfa-focDE*, *afa-draBC*, *cdt*, and *eae*. Production of CDT toxin was confirmed by phenotypic assays. Excluding *afa-draBC* and *f17*, significant differences were observed in the frequency of the virulence genes between P and NP isolates: $P <$ 1.5% for *sfa-focDE*, *eae*, and *cdt* (Table 2).

TABLE 2 Most frequently occurring serogroups and virulence genes among the 460 pathogenic and nonpathogenic isolates used for statistical analysis

Serogroup or virulence gene[a]	% Total isolates[b]	
	Pathogenic (n = 352)	Nonpathogenic (n = 108)
Serogroup		
O78*	17.6	0.9
O2*	17.3	4.6
O8	2.0	4.6
O18	9.0	5.6
O5	4.5	2.8
O1	6	3.7
Total	56.5	22.2
Virulence genes		
*sitA**	96.3	59.3
F1*	86.4	71.3
*iutA**	82.7	26.9
*tsh**	52.8	16.7
frz_{orf4}*	53.4	16.7
*tkt1**	50.6	16.7
*aec4**	46.9	13.9
P(F11)*	30.4	7.4
*aec26**	34.4	6.5
*neuC**	27.0	5.6
F17	5.7	4.6
sfa-focDE†	9.1	4.6
afa-draBC	4.0	6.5
cdt†	9.1	1.9
eae†	2.0	12.0

[a] *, Significant differences ($P < 0.001$) were observed between pathogenic and nonpathogenic strains; †, significant differences ($P < 0.015$) were observed between pathogenic and nonpathogenic strains. Strains were noted as F1 positive when both *fimA* (or the $fimAv_{MT78}$ variant) and *fimH* genes were present and P(F11) positive when the three genes—*felA*, *papC*, and a variant of *papG*—were present.

[b] For pathogenic strains, of five chicks inoculated subcutaneously, one to five died; for nonpathogenic strains, none of five chicks inoculated subcutaneously died.

Genes encoding for enterotoxins and verotoxins usually associated with intestinal pathologies in mammals, such as CNF1, LT, STa, STb, VT1, and VT2, were not detected, and no exotoxin could be detected in phenotypic assays.

First, a statistical analysis was performed using this set of 460 isolates. The statistical analysis revealed four patterns of virulence factors, each including more than 90% of the pathogenic strains: *iutA*+, P(F11)+; *iutA*+, P(F11)−, frz_{orf4}+; *iutA*+, P(F11)−, frz_{orf4}−, O78+; and *iutA*−, *aec26*+, *sitA*+ (Table 3, analysis 1). Among the 258 isolates belonging to one of these four patterns, 247 (95.7%) were pathogenic strains. Pathogenic strains were identified with an error margin of 4.3%. Since strains that killed only one chick in the lethality test are probably less virulent strains than those that killed two to five chicks, we checked whether removing them from the analysis could influence the resulting patterns.

Thus, a second statistical analysis (analysis 2) was performed using a set of 416 strains issued from the initial set of 460 strains after removal of the 44 strains that had killed only one chick. This

TABLE 3 Genetic patterns associated with *E. coli* pathogenicity in chickens

Analysis and identified genetic pattern[a]	No. of isolates	% of pathogenic isolates[b]
Analysis 1		
iutA+, P(F11)+	105	98.1
iutA+, P(F11)−, frz_{orf4}+	107	93.5
iutA+, P(F11)−, frz_{orf4}−, O78+	32	96.9
iutA−, *sitA*+, *aec26*+	14	92.9
Analysis 2		
iutA+, P(F11)+	99	98.0
iutA+, P(F11)−, frz_{orf4}+	99	92.9
iutA+, P(F11)−, frz_{orf4}−, O78+	32	96.9
iutA−, *sitA*+, *aec26*+, *aec4*+	11	90.9

[a] For analysis 1, a statistical analysis was conducted on a sample of 460 isolates, including 352 pathogenic strains (of five chicks inoculated subcutaneously, one to five died) and 108 nonpathogenic isolates (of five chicks inoculated subcutaneously, none died). For analysis 2, a statistical analysis was conducted on 416 isolates from the analysis 1 sample excluding the isolates that killed only one chicken. This group comprised 308 pathogenic strains (of five chicks inoculated subcutaneously, two to five died) and 108 nonpathogenic isolates (of five chicks inoculated subcutaneously none died).

[b] For analysis 1, "pathogenic isolates" refers to isolates where, of five chicks inoculated subcutaneously, one to five died. For analysis 2, "pathogenic isolates" refers to isolates where, of five chicks inoculated subcutaneously, two to five died.

TABLE 4 Validation of the genetic patterns A, B, C, and D

Genetic pattern[a]	No. of isolates tested	% of pathogenic isolates[b]
A	81	97.5
B	199	94.5
C	64	100
D	51	92.2

[a] Genetic patterns: A, *iutA*+, P(F11)+; B, *iutA*+, P(F11)−, frz_{orf4}+; C, *iutA*+, P(F11)−, frz_{orf4}−, O78+; D, *iutA*−, *sitA*+, *aec26*+.

[b] That is, of five chicks inoculated subcutaneously, one to five died.

second analysis revealed four patterns of virulence factors each including more than 90% of the pathogenic strains. Three corresponded to the first three patterns identified above, and the fourth one regrouped strains that were *iutA*−, *sitA*+, *aec26*+, *aec4*+ (Table 3, analysis 2). Of the 241 strains belonging to one of the four patterns, 230 (95.4%) were pathogenic strains (error margin of 4.6%).

Thus, among the 352 pathogenic isolates of the sample used in the statistical analysis, 70.2% (247/352) were identified using the four genetic patterns defined by the first analysis, and 69.3% (244/352) were identified using the four genetic patterns defined by the second analysis. Three isolates were responsible for the difference observed between both analyses since they were excluded from the fourth genetic pattern of the second analysis because they were negative for the gene *aec4*. All other isolates were identical in both analyses. In sum, both analyses yielded very similar results, and the addition of gene *aec4* in the fourth pattern did not improve the identification of pathogenic isolates.

Consequently, we decided to keep the four patterns of virulence factors identified in the first statistical analysis, which we named genetic patterns A [*iutA*+, P(F11)+), B (*iutA*+, P(F11)−, frz_{orf4}+], C [*iutA*+, P(F11)−, frz_{orf4}−, O78+), and D (*iutA*−, *sitA*+, *aec26*+). However, when a strain did not belong to one of the defined genetic patterns (A, B, C, or D), it was not possible to conclude whether such a strain was pathogenic or not since the other genetic patterns contained both pathogenic and nonpathogenic strains in proportions that were not discriminatory (see Fig. S1 in the supplemental material).

Validation of virulence patterns. The reliability of the correlation of these four virulence patterns with the pathogenicity of avian *E. coli* strains was checked. First, the presence of the 19 associated virulence genes on the remaining 1,031 isolates of the collection was detected by using PCR assays and hybridization. *E. coli* isolates belonging to genetic pattern A, B, C, or D were then selected and tested for pathogenicity using the 1-day-old chick lethality test.

The results showed that 97.5% of the *E. coli* isolates belonging to the pattern A were pathogenic strains, as well as 94.5, 100, and 92.1%, of the isolates belonging to patterns B, C, and D, respectively (Table 4). These four virulence patterns allow the identification of pathogenic avian *E. coli*, whatever the animal origin of the isolates (see Table S3 in the supplemental material). Moreover, well-studied APEC strains belong to these virulence patterns. Indeed, the APEC strains TK3 (22), BEN2908 (12), 789 (2), and BEN687 (43) belong to patterns A, B, C, and D, respectively.

DISCUSSION

It has been reported that virulence factors (VFs) could be used as detection markers for APEC. In the present study, we defined a validated PCR diagnostic assay based on four different associations of VFs that enables the identification of 70.2% of APEC. Pathogenic strains were identified with a 4.3% error margin.

The prediction of the pathogenicity of *E. coli* strains isolated from diseased animals is crucial. Routine diagnosis of avian *E. coli* isolates in veterinary laboratories is based on serotyping using commercially available serogrouping assays, which allow identification of the O1, O2, and O78 serogroups. Almost half of the strains studied here belonged to six different serogroups: O1, O2, O5, O8, O18, and O78. In the sample of 460 isolates used for statistical analysis, 223 isolates (48.5%) belonged to one of these six major serogroups, among which 199 were pathogenic strains. A diagnostic assay based on determining the major serogroups would thus only identify 56.5% (199/352) of the pathogenic strains (Table 2). However, the error margin would be 10.8% (24/223), considerably impairing the reliability of the method. Our data clearly show that the diagnostic scheme described here, which is based on identifying VFs, allows more APEC strains to be identified with a lower error margin than when using serogrouping. This is probably due to the fact that these six serogroups are shared between APEC strains and avian fecal isolates of *E. coli*, as demonstrated by Rodriguez-Siek et al. (47).

The statistical analysis leading to the PCR diagnostic protocol described here is based on a decision-tree algorithm. Decision-tree algorithms are promising tools that could improve diagnosis. For example, they have been used to predict the diagnosis and outcome of dengue fever (57). To date, several PCR multiplex assays have been described to diagnose APEC (27, 33, 55). However, each assay only described one association of VFs. Considering the extreme diversity encountered in *E. coli* species, a single set of VFs is insufficient to diagnose the majority of APEC strains. Ewers et al. developed a multiplex PCR system to detect the presence of eight VFs, revealing various combinations of five to eight VFs (27). Moreover, diagnostic tests were not validated in most of

the published studies. The only exception is a study conducted to identify the minimum number of VFs that could be used to distinguish an APEC strain from an avian fecal isolate, which showed that a subset of five genes enabled the identification of an APEC strain, although the error margin was 14.6% (33).

The panel of genes implemented in the diagnostic schemes described here includes various VFs, some of which have been well characterized. They are mainly involved in the colonization of the internal organs of chickens, via different strategies. These include two iron acquisition systems. Gene *iutA*, one of the five genes of the aerobactin operon, encodes an outer membrane protein involved in the high-affinity binding of Fe^{3+}-aerobactin and is plasmid located (32, 41) or could be also chromosomally encoded in some APEC strains (unpublished data). The aerobactin system plays a role in the persistence and generation of lesions in APEC-infected chickens (20). Gene *sitA* encodes a periplasmic binding protein of the SitABCD transport system, which is involved in iron and manganese transport and could be both chromosomally and plasmid located (41, 50, 52). SitABCD has been shown to play a role in virulence (49).

P fimbriae are known to mediate the binding of bacteria to α-D-galactopyranosil-β-D-galactopyranoside receptors. Strains expressing P fimbriae have a mannose-resistant hemagglutination phenotype (23). Genes encoding fimbriae are located on a chromosomal pathogenicity island (34). It has been shown that P fimbriae are expressed *in vivo* in the air sacs, lungs, and kidneys and in blood and pericardial fluid. No expression has been observed in the liver, spleen, or trachea. This observation in line with *in vitro* studies demonstrating that P fimbriae are not involved in the adherence of APEC to tracheal and pharyngeal epithelial cells (46, 60). It has recently been shown in an APEC strain of serogroup O1 that P fimbriae are involved in the virulence of that strain in chickens (35). Gene frz_{orf4} is chromosomally located and belongs to the *frz* operon, which encodes a phosphoenolpyruvate carbohydrate phosphotransferase system transporter and enzymes involved in sugar metabolism (48). A link between the expression of this locus and *E. coli* pathogenic abilities was confirmed by experiments showing its role in promoting bacterial fitness under stressful conditions, such as oxygen restriction or the late stationary phase of growth, and in promoting growth in chicken serum or the intestinal tract during *in vivo* competition assays. The *frz* operon also seems to be involved in the cell surface expression of F1 fimbriae, which are major adhesins of *E. coli*, via a mechanism yet to be characterized (48). Gene *aec26* is chromosomally located and encodes a putative membrane protein component of a type VI secretion system (T6SS) (52, 54). In an APEC strain, this T6SS has recently been shown to contribute to the adhesion and invasion of epithelial cells (HeLa) by influencing the expression of F1 fimbriae and to improve the virulence of the strain for 1-day-old chicks (15). O78 lipopolysaccharide contributes to serum resistance and colonization of internal organs of inoculated chicken with an APEC strain of serogroup O78 (40).

The fact that the VFs described here have been demonstrated to be involved in the pathogenic process of avian colibacillosis probably accounts for the reliability of the diagnostic method that has been developed.

Moreover, the four associations of VFs identified by the statistical analysis suggest that APEC strains use different strategies to invade the host, according to their genetic equipment, all resulting in the same pathogenic process known as avian colibacillosis.

Nevertheless, almost 30% of APEC isolates in our sample were not identified as pathogenic strains, possibly due to the existence of other APEC VFs that have not been examined here and which could improve strain discrimination. The use of newly identified VFs, for example, a novel fimbrial adhesin called EA/I (1) and the autotransporter adhesin AatA (14), or VFs not included in the present study, such as IbeA (28) and Vat (45), would help to improve the present diagnostic method.

In sum, we propose a diagnostic tool based on four associations of virulence genes that enables the identification of more APEC strains than previously described methods.

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