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Research Note—

Genotypic and Phenotypic Characterization of Potential Virulence of Intestinal Avian Escherichia coli Strains Isolated in Algeria

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SUMMARY. In order to characterize potential pathogenic *Escherichia coli* strains isolated from diarrheic hens and chickens originating from intensive battery rearing in North Algeria, the presence of a large range of virulence factors and markers was studied in 50 strains by DNA-DNA hybridization on colonies and phenotypic tests. The sequences we focused on were those coding for adhesins F5, F41, F17, Pap, Afa, and Sfa; intimin Eae; and toxins STa, STb, LT1, Stx1, Stx2, CNF1, and CNF2. The phenotypes explored were the colicins, aerobactin, hemolysins, and hemagglutinin production and serum resistance.

The genotypic and phenotypic tests enabled us to categorize the isolates into two distinct groups: those with a potential to invade the host (27 strains were serum resistant and/or produced aerobactin), among which three strains were also potentially diarrheagenic, one strain was LT1+ F17+ Afa+ Pap+ (enterotoxigenic *E. coli*) and the two others were Stx1 (verotoxigenic *E. coli*). Twenty-three strains were colicinogenic, including 19 strains producing colicin V. This latter factor was also detected in isolates negative for the other virulence factors.

On the basis of the type of erythrocytes agglutinated, we established 14 mannose-resistant hemagglutination patterns among the 37 strains tested, including 22 serum-resistant and/or aerobactin producing strains and 15 strains negative for these two characters.

None of the strains produced alpha hemolysin, whereas two strains produced beta hemolysin and enterohemolysin, respectively. Congo red fixation was observed in 25 strains. No relationship could be detected between Congo red fixation and the presence of other virulence markers, such as serum resistance and aerobactin production.

This study shows that among isolates originating from the feces of diarrheic chickens, the proportion of potentially diarrheagenic *E. coli* strains is low.

RESUMEN. *Nota de Investigación*—Caracterización genotípica y fenotípica de la virulencia potencial de las cepas intestinales de Escherichia coli aviar aisladas en Algeria.

Se estudió la presencia de factores y marcadores genéticos de virulencia en 50 cepas de *Escherichia coli* aviar, aisladas a partir de gallinas criadas en jaulas que presentaban síntomas de diarrea, con el fin de caracterizar la patogenicidad de las mismas. Se utilizaron técnicas de hibridación de ADN-ADN y caracterización fenotípica de las colonias bacterianas. Las secuencias usadas en este estudio fueron las que codifican para las adhesinas F5, F41, F17, Pap, Afa y Sfa; también fueron utilizadas las secuencias de las toxinas STa, STb, LT1, Stx1, Stx2, CNF1 y CNF2. La caracterización fenotípica incluyó la producción de aerobactina, hemolisina, hemaglutinina y la resistencia a la presencia de suero. Los estudios genotípicos y

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fenotípicos permitieron categorizar las cepas aisladas en dos grupos diferentes. El primer grupo consistió de cepas capaces de invadir las células del huésped (compuesto por 27 cepas que fueron resistentes al suero, productoras de aerobactina o ambas), dentro de las cuales hubo tres cepas con el potencial de causar diarrea en las aves. Una de estas cepas presentaba las secuencias que codifican para la toxina LT1 y para las adhesinas F17, Afa y Pap (*E. coli* enterotoxigénica), presentando las dos cepas restantes la secuencia que codifica para la producción de la toxina Stx1 (*E. coli* verotoxigénicas). Veintitrés de las cepas incluidas en este grupo fueron productoras de colicina, de las cuales 19 fueron también capaces de producir colicina V. Este último factor de virulencia fue también detectado en cepas negativas para los factores de virulencia restantes. Basados en el tipo de eritrocitos que estas cepas son capaces de aglutinar, se estableció la presencia de 14 patrones de hemaglutinación resistentes a manosa, en un grupo de 37 cepas estudiadas. Dentro de este grupo también se encontraron 22 cepas resistentes a suero y productoras de aerobactina, mientras que las 15 cepas restantes fueron negativas para ambas caracteristicas. Ninguna de las cepas estudiadas fue positiva para la producción de alfa hemolisina, mientras que dos de las cepas eran capaces de producir beta hemolisina y enterohemolisina. Veinticinco de las cepas caracterizadas fueron capaces de fijar rojo Congo. La presencia de esta característica no pudo ser correlacionada con factores de virulencia tales como la resistencia al suero o la producción de aerobactina. Este estudio demuestra que dentro de las cepas aisladas a partir de aves con diarrea, la incidencia de cepas de *E. coli* capaces de producir diarrea es baja.

Key words: poultry, *Escherichia coli*, diarrhea, virulence factors

Abbreviations: APEC = avian pathogenic *Escherichia coli*; CFA = colonization factor antigen; Col = colicin; ETEC = enterotoxigenic *E. coli*; MRHA = mannose-resistant hemagglutination; MSHA = mannose-sensitive hemagglutination; PBS = phosphate-buffered saline; TSA = tryptic–soy agar; VTEC = verotoxigenic *E. coli*

Pathogenic *Escherichia coli* cause intestinal and extraintestinal diseases in humans and animals. These strains are divided into groups on the basis of the combinations of virulence factors and clinical specificity.

The most frequently encountered clinical manifestation of colibacillosis in poultry is septicemia, which is one of the principal causes of economic loss in the poultry industry (34,62).

Infection most often occurs via the respiratory route (28,34). The intestinal tract, although not considered to be the main portal of entry for invasive colibacillosis, represents a reservoir for avian pathogenic *E. coli* (APEC) strains, and 10%–15% of intestinal *E. coli* isolates belong to the main pathogenic O serogroups (10,34,41).

APEC usually belong to the O1, O2, and O78 serogroups (9,12,21,33). Their virulence-associated properties include production of colicin (Col)V and of adhesins, iron-chelator systems, K1 capsular antigen, serum resistance, and cytotoxic effects (17,20,22,25,37,61). The adhesins characterized to date, particularly for isolates belonging to the three O serogroups mentioned above, include mannose-sensitive hemagglutinating F1A or F1A-like fimbriae (4,19,35), whose role in pathogenicity has been established (20); mannose-resistant hemagglutinating P and P-like fimbriae (1,21,48,51,57), whose role in pathogenesis remains to be established (23); and nonhemagglutinating AC/1 (avian *E. coli* 1) adhesin (64). They also include protein filaments referred to as curli and temperature-sensitive hemagglutinin (47), whose role in pathogenesis also remains to be established (18). The molecular methods used demonstrate that novel virulence factors are also present (11).

However, the association between *E. coli* and enteritis in chickens is still controversial despite some cases documented in the Philippines (38) and in North America (24), although the virulence factors of intestinal APEC have not been studied in depth. The production of enterotoxins (STa and LT) typical of enterotoxigenic *E. coli* (ETEC) (52,53) and the presence of receptors for STa toxin in the digestive tract of chickens have been described (39). Some APEC isolated from the digestive tract also produce cytotoxins (25,27), but there are no reports dealing with the presence of specific fimbrial adhesins associated with intestinal APEC to date.

The purpose of this work was thus to determine the presence and the relative importance of potential virulence factors and markers of *E. coli* isolates from the feces of diarrheic hens and chickens from intensive battery-rearing units in Algeria by phenotypic and genotypic assays. This will enable us to compare Algerian strains with those isolated from developed countries and to establish the possibility that these isolates are involved in diarrhea and subsequent invasion of the host.

MATERIALS AND METHODS

Bacterial strains. The 50 isolates examined in this study originated from the collection at the Laboratoire de Microbiologie, Ecole Vétérinaire d'Alger (Dr. Mohamed Ou Said). They were isolated between 1990 and 1996 from the feces of diarrheic hens and chickens 3–10 wk of age originating from intensive battery-rearing units in North Algeria.

Aerobactin production. Aerobactin production was determined after growth of bacteria in liquid media by the Csaky test (14) modified by Der Vartanian (15).

Colicin production. Colicin production was evaluated by the double-layer method (30). The indicator strains were *E. coli* XA, sensitive to most of the colicins, and its chromosomal mutant *E. coli* XA ColV^R, resistant to colicin V.

Resistance to the bactericidal effect of serum. Bacterial resistance to serum was determined by the "spot" technique (29). Tryptic–soy agar (TSA) medium was sheet seeded with bacterial culture in logarithmic growth phase diluted 1/500. After drying at 37 C for 15 min, one drop of fresh undiluted sheep serum (50 µl) was added to the medium. The presence or absence of a zone of growth around the serum spot after overnight incubation at 37 C was noted.

α, β hemolysin and enterohemolysin production. Hemolysin production was examined after growing bacteria on Columbia agar supplemented with 5% fresh defibrinated sheep blood or in the same medium containing an equivalent quantity of sheep blood washed three times in phosphate-buffered saline (PBS), pH 7.4.

α and β hemolysins induced a clear lysis zone on both media after incubation at 37 C for 3 hr and 24 hr, respectively. The lysis zone for β hemolysin is clearer than that for α hemolysin. Lysis due to enterohemolysin was visible after overnight incubation at 37 C only on Columbia agar medium supplemented with washed sheep erythrocytes (6).

Congo red fixation. To evaluate the fixation of Congo red, bacteria were grown at 37 C for 24 hr on TSA supplemented with 0.02% Congo red. Positive colonies appeared red, whereas negative colonies appeared pale.

Hemagglutination. Hemagglutination tests were performed in 96-Well plates. A volume of 50 µl of dense bacterial suspension (10^{10} colony-forming units/ml in PBS, pH 7.4), obtained from an overnight culture at 37 C in colonization factor antigen (CFA) medium (26), was added to 50 µl of a 3% erythrocyte suspension in either PBS alone or PBS containing 4% D-mannose.

The plates were gently rocked, then incubated at room temperature for 5 min and at 4 C for 5 min. The result was noted after several incubations at room temperature and at 4 C (30–45 min). The hemagglutination was considered mannose-resistant (MRHA) if it occurred in the presence of mannose and mannose-sensitive (MSHA) if it was inhibited by mannose.

The hemagglutination test was performed with human (A+, B+, O+), bovine, avian, and sheep erythrocytes.

Gene probes. Gene probes for the different adhesins and toxins were derived from *E. coli* K12 containing the appropriate recombinant plasmid or after specific polymerase chain reaction amplification from a wild-type or recombinant plasmid (44,45,46) (Table 1). Radioactive labeling was achieved by the multiprime method with a random priming DNA labeling kit (Boehringer Mannheim, Bruxelles, Belgium). The reference strains for the different gene probes were 431 (STa+ F5+ F41+); G7 (STb+ LT+); E2348/69 (Eae+); 211 (Stx2+); 1625 (Eae+ Stx1+); J96 (CNF1+ Pap+ Sfa+); 1676 S (CNF1+ Sfa+); 1565 S (CNF2+ F17+), A30 (Afa+); 25KH (F17+ Pap+).

Strain HS was used as a negative control for all probes.

DNA-DNA hybridization of colonies. The strains were spotted onto Mueller Hinton agar. After overnight incubation at 37 C, colonies were transferred to Whatman 541 filter paper, which was then processed and hybridized overnight with DNA probes in 3× sodium chloride–sodium citrate at 65 C without formamide (47). Hybridization conditions allowed for 15%–20% mismatch.

RESULTS

DNA-DNA colony hybridization of the 50 strains with the 16 different gene probes revealed that two strains hybridized with the probes for Stx1 toxin (verotoxigenic *E. coli* [VTEC]) and one strain was positive with specific probes for LT1 toxin, and Pap, Afa, and F17 adhesins (ETEC). Forty-seven strains did not hybridize with any of the gene probes used (Table 2).

Table 1. Derivation of the various gene probes.

Name	Gene probe: Species of origin (strain)	Size (bp)	Specificity	Recombinant plasmids: Name	Enzyme(s)[A]
STa	Porcine (MM294)	157	STa toxins	pRIT10130	*Hin*f1
STb	Porcine (G7)	278	STb toxins	pRAS1	PCR
LT1	Porcine (P307)	800	LT1 toxins	pEWD299	*Hin*d2
F5(K99)	Bovine (B41)	1700	F5 adhesins	pFK99	*Hpa*1
F41	Bovine (VAC1676)	700	F41 adhesins	pDGA17	*Pst*1 + *Hin*d2
Eae	Human (E2348/69)	1000	Intimins	pCVD434	*Sal*1 + *Kpn*1
Stx1	Human (933)	1142	Stx1 toxins	pJN37-19	*Bam*H1
Stx2	Human (933)	842	Stx2 toxins	pNN11-19	*Pst*1
CNF1/2	Ovine (S5)	335	CNF1/CNF2 toxins	pEOSWO1	*Pst*1 + *Cla*1
CNF2	Ovine (S5)	875	CNF2 toxins	pEOSWO3	*Xho*1 + *Pst*1
Pap	Human (C1212)	800	P fimbriae	pDAL201B	*Sma*1 + *Kpn*1[B]
Sfa	Human (536)	410	S fimbriae	pANN801B	PCR
F17	Bovine (25KH)	1200	F17 fimbriae	pPLHD2	*Hin*d3 + *Bam*H1
Afa	Human (A30)	750	Afa adhesins	—[C]	PCR

[A]Restriction endonucleases were used to derive the gene probes from the vector plasmids.
[B]*Sma*1 enzyme allows the production of a fragment of 1800 bp, which is restricted with *Kpn*1.
[C]The wild strain A30 was used for amplification.

Of 50 strains tested, six strains, including the ETEC strain, produced aerobactin, and 23 strains, including the three VTEC and ETEC strains, were resistant to the bactericidal effects of serum. Among 23 strains that were colicinogenic, 19 produced ColV. Fourteen ColV+ strains were among the serum-resistant and/or aerobactin-producing strains (Table 2). Several combinations of these virulence properties were noted: 10 strains were ColV+ and serum resistant, two strains were ColV+ and produced aerobactin, and two strains were simultaneously serum resistant and produced aerobactin and colicin V (Table 2).

Thirty-seven strains, including 22 serum-resistant and/or aerobactin-producing strains and 15 strains negative for these two characters, were tested for their ability to agglutinate erythrocytes from different animal species. Eleven strains, including six serum-resistant strains, had a MSHA+/MRHA+ phenotype, and 22 strains, including 12 serum-resistant and/or aerobactin-producing strains, had only a MRHA+ phenotype. Four strains did not agglutinate any of the erythrocytes used (Table 3). On the basis of the type of erythrocytes agglutinated, 14 MRHA patterns were established (Table 3).

None of the strains produced alpha hemolysin, although one strain produced a beta hemolysin and another strain produced an enterohemolysin (Table 2).

Congo red fixation was observed in 25 strains (Table 2). No correlation could be established between this property and any of the other virulence markers studied, notably serum resistance and aerobactin production.

DISCUSSION

The involvement of intestinal *E. coli* in avian diarrhea has not been established, nor has their role in septicemia or systemic disease. With this aim in view, we investigated the presence of some virulence factors and markers among a collection of *E. coli* strains isolated from the feces of hens and chickens originating from intensive battery rearing in Algeria.

Many authors have classified *E. coli* strains into groups according to the presence of specific virulence factors. Serum resistance and the aerobactin iron-sequestering system are frequently associated with *E. coli* causing extraintestinal disease in humans and in other animals (43). Dozois *et al.* (21) suggested that the aerobactin system is important in the pathogen-

Table 2. Distribution of virulence markers and virulence factors of avian *E. coli* strains.^

Strain	Hybridization with gene probe for		aer	Serum resistance	CR binding	Col	Hemolysin
	Toxin(s)	Adhesin(s)					
V63	LT1	F 17, Afa, Pap	+	+	+	ColV+	–
SA5	Stx1	–	–	+	–	–	–
V152	Stx1	–	–	+	–	ColV+	–
SA18	–	–	–	+	–	–	–
SA2	–	–	–	+	++	ColV+	Ent
SA8	–	–	–	+	++	–	–
SA6	–	–	–	+	+	ColV+	–
SA7	–	–	–	+	+	–	–
V107	–	–	–	+	–	Col+	–
SA15	–	–	–	+	–	–	–
SA12	–	–	–	+	++	ColV+	–
V27	–	–	–	+	+	ColV+	–
V102	–	–	–	+	–	–	–
V127	–	–	+	+	–	ColV+	–
V172	–	–	–	+	–	ColV+	–
P2S2	–	–	–	+	–	–	–
V34	–	–	–	+	+	ColV+	–
V164	–	–	–	+	–	ColV+	–
SA1	–	–	–	+	++	ColV+	–
V109	–	–	–	+	+	–	–
V161	–	–	–	+	–	Col+	–
V168	–	–	–	+	–	ColV+	–
V163	–	–	–	+	–	Col+	–
V101	–	–	+	–	–	–	–
V122	–	–	+	–	–	ColV+	–
V52	–	–	+	–	+	ColV+	–
V131	–	–	+	–	–	–	–
SA14	–	–	–	–	++	–	–
SA11	–	–	–	–	–	–	–
SA16	–	–	–	–	+	–	–
SA3	–	–	–	–	++	–	–
P2S1	–	–	–	–	–	–	–
SA13	–	–	–	–	–	–	–
SA4	–	–	–	–	++	ColV+	–
V166	–	–	–	–	–	ColV+	–
V119	–	–	–	–	++	–	–
V121	–	–	–	–	+	–	–
P1SC2	–	–	–	–	++	–	–
V173	–	–	–	–	+	ColV+	–
P2S3	–	–	–	–	++	Col+	–
P1S3	–	–	–	–	++	–	β
SA17	–	–	–	–	–	–	–
V171	–	–	–	–	–	ColV+	–
V128	–	–	–	–	–	ColV+	–
V162	–	–	–	–	++	–	–
V113	–	–	–	–	++	–	–
SA9	–	–	–	–	++	–	–
P1SC1	–	–	–	–	++	–	–
V108	–	–	–	–	–	–	–
V156	–	–	–	–	–	–	–

^Col = colicin; ColV = colicin V; Col+ = colicins other than ColV; aer = aerobactin production; – = negative test; + = positive test; Ent = enterohemolysin; β = beta hemolysin; CR = Congo red.

Table 3. Mannose-resistant hemagglutination patterns of avian fecal *E. coli* strains used in this work with different types of erythrocytes.[A]

Strain	MRHA pattern	MSHA pattern	Other virulence markers/factors
SA1		–	SR CR ColV+
V109		–	SR CR
SA4	H(O+A+B+), BO, OV, AV	–	CR ColV+
V121		–	CR
V171		–	ColV+
V113	H(O+A+B+), BO, OV	–	CR
V63		–	LT1+ F17+ Afa+ Pap+, aer+ SR CR ColV+
SA5		BO	STx1+, SR
SA6	H(O+A+B+), OV, AV	BO	SR CR ColV+
SA7		BO	SR CR
V119	H(O+A+B+), BO	OV	CR
V161		–	SR Col+
SA12	H(O+), OV	–	SR CR ColV+
V27	H(O+), AV	–	SR CR ColV+
SA9	H(A+), AV	H(O+), BO, OV	CR
SA11	H(A+), OV, AV	H(B+)	–
V107	BO, OV, AV	–	SR Col+
V172	BO, OV	H(O+, A+)	SR ColV+
V173		H(O+, A+, B+)	CR ColV+
V128	BO, AV	–	ColV+
SA17		–	–
SA13	OV, AV	–	–
V101		–	aer+
SA18		–	SR
V166	OV	–	ColV+
V162		–	CR
V52		–	aer+ CR ColV+
V163		–	SR Col+
V34	AV	–	SR CR ColV+
V102		–	SR
SA15		OV	SR
SA2		H(B+), OV	SR CR ColV + Ent
SA3		H(B+), OV	CR
V156		–	–
V122	–	–	aer+ ColV+
V127		–	aer+ SR ColV+
V152		–	STx1+, SR ColV+

[A]H = human erythrocytes, BO = bovine, OV = ovine, AV = avian, SR = serum resistance, CR = Congo red binding, ColV+ = colicin V, Ent = enterohemolysin, – = negative test, aer = aerobactin production.

esis of *E. coli* septicemia in chickens and turkeys, and Wooly *et al.* (61) determined that complement resistance was highly associated with septicemic isolates from chickens.

On the basis of the genotypic and phenotypic results obtained, we have classified the isolates into two groups: those with a potential to invade the host (27 strains), among which three

strains are also potentially diarrheagenic, and other strains (23 strains) (Table 2). Strains with a potential to invade the host (27 strains) were principally characterized by their ability to resist the bactericidal effect of serum and/or to produce aerobactin, both of which confer a potential for extraintestinal infection, but *in vivo* tests are required to confirm the virulence of these strains. Other characters, such as colicin synthesis and the presence of MSHA and/or MRHA adhesins, may be associated with these strains and may contribute to their pathogenicity.

The proportions of strains that were serum resistant (46%), produced aerobactin (12%), and were colicinogenic (ColV+) (38%) in this study are inferior to the proportions reported among septicemic and cellulitic strains but similar to or greater than those reported among healthy chicken strains with respect to serum resistance (42,49,61). Although the role of colicins in bacterial virulence is still debated (2,3,7,54,61), the prevalence of ColV among serum-resistant and/or aerobactin-producing strains indicates that this phenotype may constitute a virulence marker. In fact, these phenotypes can be encoded by genes located on the same plasmid, named pColV (43,63).

Our results also confirm that the great majority of avian *E. coli* strains are nonhemolytic (4,21,25,27). Only two of the isolates in this study produced a beta hemolysis and an enterohemolysis, respectively.

Congo red binding has also been proposed as a potential virulence marker by a number of authors (5,13). Our study does not indicate any specific association between Congo red fixation and the other virulence phenotypes examined. Similarly, Ike *et al.* (37) did not detect any correlation between Congo red binding and serum resistance, aerobactin production, or virulence *in vivo*. Our results thus suggest that other virulence factors are present, and Hammer *et al.* (36) have reported a correlation between Congo red binding and curli synthesis, although we did not evaluate the latter in this study.

The presence of adhesin- and toxin-encoding genes was evaluated by DNA-DNA hybridization and hemagglutination tests. Only one strain hybridized with three gene probes specific for Pap, F17, and Afa adhesins. Since this work was completed, F17- and afa-positive strains have been detected in APEC from Europe, and the *afa*-like sequences have been identified to the *afa-VIII* variant (32,40,56). The lack of hybridization with an SFA probe reported by Dozois *at al.* (21) is also in accordance with our results. Although our strains were all Eae-negative, some authors (31,56) have isolated attaching and effacing avian *E. coli* strains.

MRHA was detected in 89% of strains tested, including the MSHA+ strains. Fourteen different MRHA profiles were detected. This diversity in MRHA profiles suggests the presence of adhesins other than P-like fimbrial adhesins previously described in avian strains.

With respect to the presence of toxins, hybridization with specific DNA probes revealed that the gene for LT1 toxin was present in one strain (ETEC) and the gene for Stx1 verotoxin was detected in two strains (VTEC).

Several authors have reported the production of toxins by avian *E. coli* strains (8,25,27,58). The presence of the LT- and Stx-encoding genes in our avian strains does not necessarily imply an *E. coli* etiology for avian enteritis, but their presence may support the theory that the avian intestine acts as a reservoir for bacteria pathogenic to both humans and animals.

On the other hand, all of our strains were ST- and CNF-negative. Only one study (53) has demonstrated the production of STa in a single strain isolated from a chicken. Similarly, CNF has been found in strains isolated from various animals but has not been detected in avian strains (8,16,50).

In addition to the group of potentially invasive strains, we detected 23 strains that were not serum resistant, did not produce aerobactin, and did not present any known adhesins or toxins. However, some of them were positive for Congo red binding, colicin V production, and MRHA and/or MSHA. The presence of these factors does not necessarily imply pathogenicity because other factors are also required. On the other hand, the MRHA adhesins may confer an ability to colonize the intestine, which may permit these strains, as members of the commensal flora, to act as a defense barrier against colonization by pathogenic bacteria (55,59,60).

This study shows that, even in strains originating from the feces of diarrheic birds, the proportion of potentially diarrheagenic strains is low. This finding supports the argument that *E. coli* is not involved in avian enteritis but that

the avian intestine may be a reservoir for strains pathogenic for humans and other animals. The relative proportion of phenotypes associated with potentially invasive strains found in our study corresponds to those commonly observed in stool of healthy chickens in other countries. This indicates that *E. coli* strains isolated from chickens with diarrhea are no more or less likely to carry virulence factors than those isolated from healthy chickens.

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