Characterization of *Escherichia coli* Isolated from Cases of Avian Colibacillosis

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ABSTRACT

Forty-four western Canadian isolates of *Escherichia coli* associated with colibacillosis of turkeys and chickens were examined for serotype, antibiotic resistance, and production of aerobactin. The isolates belonged to fourteen O serogroups, with 39% of the strains being nontypeable. A high frequency of resistance to tetracycline, kanamycin, neomycin, cephalothin, streptomycin and erythromycin was observed. Most isolates produced aerobactin. Ten *E. coli* belonging to serogroups O1, O2 and O78 were also examined for pili production, hemagglutination, serum sensitivity, production of iron-regulated outer membrane proteins (IROMPS), and virulence. All isolates examined produced pili, exhibited mannose-sensitive hemagglutination of avian red blood cells and produced IROMPS under iron-restricted growth conditions. The five isolates of serogoup O1 and O2 were resistant to killing by turkey serum and were highly virulent. Only two of the five isolates of serogroup O78 were serum resistant. No correlation between serum resistance and virulence was observed in serogroup O78.

RÉSUMÉ

Quarante-quatre isolats d'*Escherichia coli* associés à la colibacillose chez la dinde et le poulet, et provenant de l'Ouest canadien, ont été examinés pour le sérotype, la résistance aux antibiotiques, et la production d'aérobactine. Les isolats appartenaient à quatorze sérogroupes O, et 39 % des souches n'étaient pas typables. Une fréquence élevée de résistance à la tétracycline, kanamycine, néomycine, céphalothine, streptomycine et érythromycine fut observée. La majorité des isolats produisait de l'aérobactine. Dix *E. coli* appartenant aux sérogroupes O1, O2 et O78 ont aussi été examinés pour la production de pili, l'hémagglutination, la sensibilité au sérum, la production de protéines de membrane externe régulées par le fer (IROMPS) et la virulence. Tous les isolats examinés ont produit des pili, ont induit une hémagglutination sensible au mannose et produit des IROMPS dans un environnement limité en fer. Les cinq isolats des sérogroupes O1 et O2 étaient résistants au sérum de dinde et étaient très virulents. Seulement deux des cinq isolats du sérogroupe O78 étaient résistants au sérum. Aucune corrélation entre la résistance au sérum et la virulence n'a été démontrée. (*Traduit par Dr Martine Bouliane*)

INTRODUCTION

Escherichia coli infection in turkeys and chickens is manifested in several forms, the most common being colibacillosis. This disease is characterized in its acute form by septicemia resulting in death and in its subacute form by airsacculitis, pericarditis, and perihepatitis (1). Such infections frequently develop as a secondary event subsequent to mycoplasma or viral infection (2). Many *E. coli* isolates commonly associated with colibacillosis in poultry belong to serogroups O1, O2 and O78 (3,4,5). Several bacterial properties, including adhesiveness and iron acquisition mediated by the aerobactin system, have been associated with virulence (6,7,8). We have characterized 44 *E. coli* strains isolated from diseased chickens and turkeys in western Canada. The strains represent a wide range of serogroups. This information will be useful when selecting *E. coli* strains suitable for use in the development of a vaccine which will be effective in the control of colibacillosis of turkeys and chickens.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTIVATION

The 44 strains used here were isolated from turkeys or chickens between three to ten weeks of age with signs of colibacillosis. The strains were collected between 1986 and 1990 from various locations in western Canada (Table I) and stored at −70°C in 25% glycerol. The isolates were routinely grown on Brain Heart Infusion medium (BHI) at 37°C. The *E. coli* K-12 strain LG1522 (*ara fepA lac leu mtl proC rpsL supE tonA trpE xyl* ColV-K30*iuc*) was obtained from Dr. J. B. Neilands, University of California, Berkeley. M9 Minimal Salts medium was prepared according to Miller (9). Tryptic soy agar (TSA) plates containing 5%

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This work was supported by grants from the Alberta Turkey Growers' Marketing Board, British Columbia Turkey Marketing Board, Manitoba Turkey Producers' Marketing Board, Saskatchewan Turkey Producers' Marketing Board, Canadian Turkey Marketing Agency and the Alberta Agricultural Research Institute.

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Submitted August 10, 1992.

sheep blood were supplied by GibMar Industries Inc., Ardrossan, Alberta. Bacteriological media and chemicals were obtained from Difco Laboratories (Detroit, Michigan) and Sigma Chemical Co. (St. Louis, Missouri).

SEROTYPING

The identification of O somatic antigens and H flagellar antigens was performed at the *E. coli* Reference Center, Pennsylvania State University, University Park, Pennsylvania.

BIOLOGICAL ASSAY FOR AEROBACTIN PRODUCTION

The production of aerobactin was determined by crossfeeding *E. coli* LG1522 (10). This mutant is deficient in enterochelin synthesis and uptake as well as aerobactin synthesis, but it expresses the receptor for the ferric-aerobactin complex. Lawns of LG1522 (10^7 colony forming units (CFU) per plate) were spread onto M9 minimal agar (9) containing 0.5% casamino acids, 40 μg/mL tryptophan and 200 μM 2,2'-dipyridyl. The strains of *E. coli* to be tested were grown for 18 hours in BHI broth at 37°C, washed once and diluted in normal saline to 1×10^7 CFU/mL. Strains were then spotted onto the lawn of indicator bacteria. Aerobactin production was detected as a halo of satellite growth after overnight incubation at 37°C.

HEMOLYSIN ASSAY

Plates of TSA containing 5% sheep blood were streaked with overnight cultures and examined for clear zones of erythrocyte lysis after 20 hours of incubation at 37°C.

ANTIBIOTIC SENSITIVITY

Antibiotic sensitivity was tested with antibiotic disks (Difco) according to standard procedures on Mueller-Hinton agar plates (11).

SERUM RESISTANCE

Bacterial resistance to the bactericidal activity of 50% serum was tested by enumerating the survivors after exposure for various intervals to turkey serum as described by Taylor (12). Turkey serum was obtained and stored at −20°C for not more than a month before use. For each experiment a pool of sera from five turkeys was used, and each strain was tested using two different pools of sera. The bactericidal effect of serum was unaltered by storage of the sera at −20°C for up to a month. The serum was tested for the presence of antibodies against serogroups O1, O2 and O78 by slide agglutination. Only sera negative for these antibodies were used. Serum sensitivity of bacteria was defined as a 2-log decrease in bacterial population after three hours of incubation in 50% serum.

TABLE I. Serotypes of *Escherichia coli* isolates

Serotype	Number of isolates from turkeys	Serotype	Number of isolates from chickens
O2:H9	1	O1:(-)	2
O2:NM	1	O2:(-)	1
O4:NM	1	05:NM	1
O36:(-)	1	O9:NM	1
O75:H42	1	O36:NM	1
O78:NM	2	O78:(-)	1
O78:(-)	2	O85:NM	3
O85:NM	1	O32/83:NM	1
O86:NM	1	Nontypeable	6
O87:H16	1		
O106:(-)	1		
O17/106:A	1		
Nontypeable	11		
Rough	2		

Key to symbols: NM = nonmotile, (-) = no reaction, A = autoagglutination

PILI PRODUCTION

Bacteria were grown in BHI without agitation and were examined after 18 and 72 hours for the presence of pili. After negative staining with 1% uranyl acetate, the pili were examined using a Philips EM 400 microscope (13).

HEMAGGLUTINATION TESTS

Escherichia coli was grown as described for pili production, washed twice, and resuspended in phosphate-buffered saline (PBS, pH 7.4). Erythrocytes were collected from heparinized turkey blood, washed three times and diluted in PBS to a final concentration of 2% (vol/vol). Erythrocytes were mixed with bacterial suspension at a ratio of 1:1 (vol:vol) on glass slides, which were cooled on ice. Hemagglutination was recorded after the slides were gently rotated for 3 min on ice. Mannose sensitivity of hemagglutination was determined by adding 0.5% D-mannose to the bacterial cell suspension before the addition of the erythrocytes.

PREPARATION OF OUTER MEMBRANE (OMP) ENRICHED FRACTIONS

Outer membranes were prepared from cells grown under iron-restricted and iron-replete conditions. Cells were grown in BHI broth with or without 2,2'-dipyridyl at a final concentration of 200 μM. An overnight culture was diluted 1:100 and grown with shaking at 37°C for four hours. The absorbance at 660 nm was approximately 1.5. The OMP preparations were prepared after extraction with 2% (wt/vol) sarcosyl (sodium N-lauroyl sarcosin) (14). Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% gels (15) and stained with Coomassie brilliant blue R-250.

PATHOGENICITY TESTING

The pathogenicity of *E. coli* isolates was evaluated in one-day-old chickens, obtained from commercial sources. Six birds were inoculated subcutaneously with 0.25 mL of BHI containing 10^4 or 10^6 CFU of each isolate. The actual dose was determined by viable cell counts of the inoculum. Birds were maintained for seven days postinoculation and monitored daily for mortality. The guidelines in the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care were followed throughout the study.

RESULTS

Of the 44 *E. coli* isolates obtained from turkeys and chickens, 23% belonged to serogroup O1, O2 or O78. Thirty-nine percent of the isolates were nontypeable. The remaining 15 isolates belonged to ten different serogroups with only serogroup O85

TABLE II. *In vitro* antibiotic sensitivity of avian *E. coli* isolates

Antibiotic	Disc potency (μg)	% resistant
Tetracycline	30	80
Chloramphenicol	30	2
Kanamycin	30	68
Ampicillin	10	14
Neomycin	30	80
Cephalothin	30	68
Streptomycin	10	95
Erythromycin	15	98

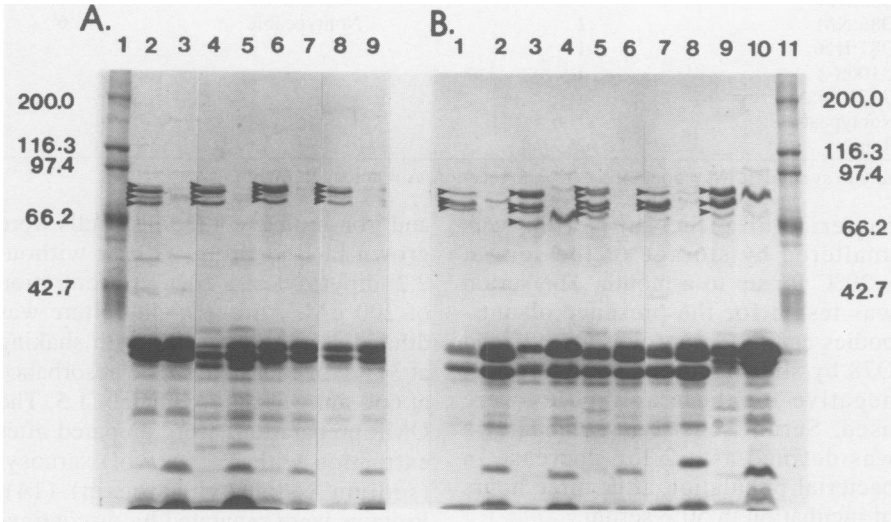

Fig. 1. Iron-regulated outer membrane proteins produced by avian isolates of *E. coli* of serotypes O1, O2 and O78. Cultures were grown in BHI broth without addition (iron replete) or in BHI broth plus 200 μM 2,2'-dipyridyl (iron restricted). A. Serotype O78 isolates. Lane 1, molecular weight markers (myosin, 200,000; β-galactosidase, 116,300; phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000). EC343 grown under iron-restricted (lane 2) and iron-replete (lane 3) conditions: EC101 grown under iron-restricted (lane 4) and iron-replete (lane 5) conditions; EC223 grown under iron-restricted (lane 6) and iron-replete (lane 7) conditions; EC226 grown under iron-restricted (lane 8) and iron-replete (lane 10) conditions. B. Serotype O1 isolates. EC222 grown under iron-restricted (lane 1) and iron-replete (lane 2) conditions; EC334 grown under iron-restricted (lane 3) and iron-replete (lane 4) conditions. Serotype O2 isolates. EC317 grown under iron-restricted (lane 5) and iron-replete (lane 6) conditions; EC230 grown under iron-restricted (lane 7) and iron-replete (lane 8) conditions; EC104 grown under iron-restricted (lane 9) and iron-replete (lane 10) conditions. Lane 11, molecular weight markers.

being represented by more than two isolates (Table I). Three of the four isolates of serogroup O85 were obtained from the same geographic region within a four-month period and may represent several isolations of the same strain.

A high level of resistance to antibiotics was observed (Table II). Many isolates exhibited resistance to more than one antibiotic, but no clear pattern of resistance was observed. Chloramphenicol and ampicillin were the only two antibiotics tested to which the majority of the *E. coli* isolates were sensitive.

Production of aerobactin, as determined by bioassay, was detected in 95% of the avian *E. coli* isolates. The two strains that failed to produce aerobactin were of different O serogroups. None of the strains tested appeared to produce hemolysin detectable on sheep blood agar.

Reports in the literature indicate that serogroups O1, O2 and O78 make up a significant portion of the isolates implicated in colibacillosis (3,4,5). Therefore the ten isolates belonging to these serogroups were characterized in more detail. All ten isolates were heavily piliated after 72 hours of stationary culture. Only one isolate belonging to serogroup O2 was heavily piliated after 18 hours of growth. In addition, all isolates tested were able to agglutinate turkey erythrocytes. This agglutination was mannose sensitive.

All isolates of serogroup O78 tested produced four iron-regulated outer membrane proteins (IROMPS) when grown under iron-limiting conditions. These proteins had apparent molecular weights of 84, 81, 77 and 75 kDa (Fig. 1A). Both isolates of serogroup O1 also produced four IROMPS, but differences were observed in the size and relative amounts of the proteins produced. EC222 produced IROMPS with apparent molecular weights of 83, 77, 76 and 74 kDa while EC334 produced IROMPS of 83, 77, 74 and 70 kDa. Two serogroup O2 isolates, EC230 and EC104, produced three IROMPS. They shared common 83 and 76 kDa proteins, but EC104 produced a protein of 71 kDa while EC230 produced a protein of 74 kDa. The third serogroup O2 isolate, EC317, produced five IROMPS with apparent molecular weights of 86, 83, 76, 74 and 71 kDa. Such qualitative and quantitative variation in the IROMPS of other pathogenic *E. coli* has been reported (16,17).

Only two of the five serogroup O78 isolates appeared to be resistant to killing by serum (Fig. 2A). The serum sensitive isolates of serogroup O78 showed some variation in the time required for killing to occur, but in all cases, after three hours of incubation less than 1% of the bacterial cells remained viable. In contrast, all *E. coli* of serogroups O1 and O2 were resistant to killing by 50% turkey serum (Fig. 2B).

Escherichia coli EC317 had been previously shown to be highly virulent in a young turkey model. When evaluated using the newborn chick model, all other isolates of serogroups O1 and O2 appeared to be as virulent as EC317. Greater variation was observed in the virulence of the strains belonging to serogroup O78 (Table III) and as a group, they appeared less virulent than the serogroup O1 and O2 isolates. For example EC101 and EC102 appeared less virulent than EC317, as challenge with these two strains resulted in few mortalities even when 40 times as many bacteria were used. No correlation was noted between serum

resistance and virulence in the serotype O78 strains (Table III).

DISCUSSION

A large number of serogroups were represented in the avian *E. coli* isolates studied, similar to the diversity observed by other researchers in central Canada and elsewhere (3,18,19). Dozois *et al* (8) found that among 112 isolates of *E. coli* from cases of colibacillosis in Quebec, 16 serogroups were represented. The 93 *E. coli* isolates from cases of avian colibacillosis in Spain, which were examined by White *et al* (18) represented 25 serogroups, while the 79 *E. coli* strains isolated in the USA were made up of 18 O serogroups (19). It has previously been reported that serogroups O1, O2 and O78 are the predominant serogroups involved in colibacillosis. In our study serogroup O78 was the most common serogroup isolated (10%) followed by serogroup O85. The occurrence of serogroup O85 may be overrepresented as three of the four isolates were obtained from the same laboratory within a four-month period. The involvement of a particular serogroup in disease appears to vary with location. For example Whittam *et al* (19) reported that serogroup O78 made up 5% of isolates examined while serogroup O2 contributed 56%. In contrast Dozois *et al* (8) in a study of 83 *E. coli* isolates from diseased chickens and 29 isolates from diseased turkeys from Quebec observed that 52% of the isolates from diseased chickens and 24% of the isolates from diseased turkeys were serogroup O78. Serogroup O2 was found in 2.4% of the chickens and was not isolated from the group of turkeys. The presence of a large proportion of untypeable strains was a common characteristic of all groups of *E. coli* from avian sources regardless of geographic location. The observation of large variations in contribution of specific serogroups, the wide variety of serogroups isolated, and the large number of untypeable isolates make it difficult to produce a meaningful classification of *E. coli* pathogenic for avian species based on serogroup alone. Pathogenic avian *E. coli* have recently been classified into various clonal groups using multilocus enzyme electrophoresis (18,19). This approach may produce a system of grouping these isolates that more closely reflects the genetic diversity present as it has been shown that isolates of different serogroups may be closely related (20).

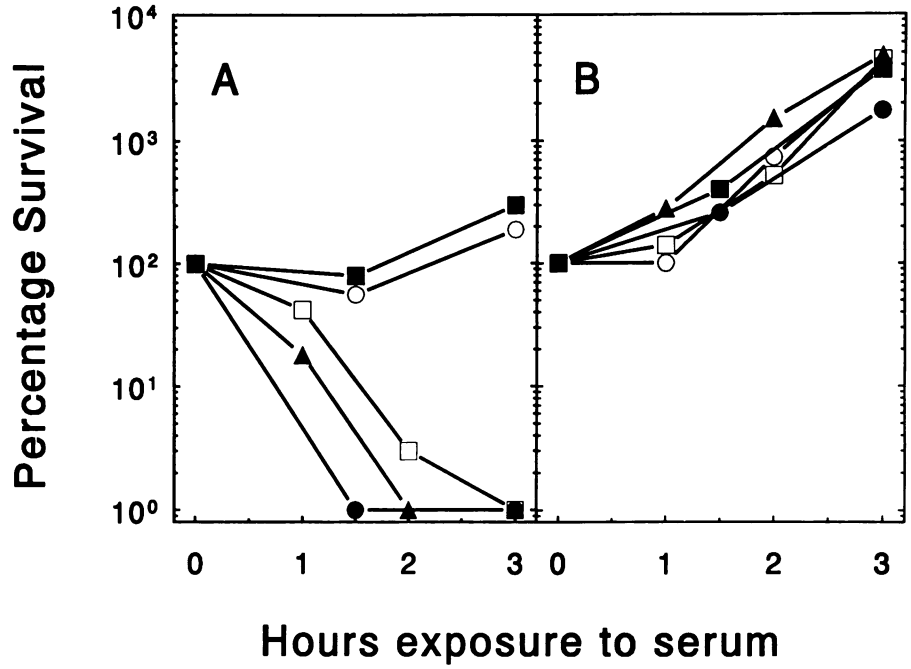

Fig. 2. Response of avian *E. coli* strains to normal turkey sera. A. Serotype O78; EC(101) (■), EC102 (○), EC226 (□), EC343 (●) and EC223 (▲). B. Serotype O1; EC222 (□) and EC334 (■). Serotype O2; EC317 (○), EC230 (●) and EC104 (▲).

TABLE III. Pathogenicity of *E. coli* of avian origin for chicks

Strain	Serotype	Dose × 10^4 CFU	Mortality after seven days
—	—	—	0
EC317	O2	1.9	4
EC230	O2	2.1	5
EC104	O2	2.3	6
EC222	O1	1.4	5
EC334	O1	2.1	4
EC226	O78	1.6	3
EC223	O78	2.6	3
EC223[a]	O78	90.0	3
EC343	O78	1.9	3
EC343[a]	O78	97.0	6
EC101	O78	2.4	2
EC101[a]	O78	85.0	0
EC102	O78	2.1	1
EC102[a]	O78	85.0	0

All groups contained six birds except the control group which contained only five birds. All isolates except EC223, EC226 and EC343 were serum resistant
[a]These data were collected during a separate experiment

A high rate of antibiotic resistance was observed in the isolates tested. This has been previously reported for other groups of avian isolates of *E. coli* (3,21) and probably results from the extensive use of antibiotics in the poultry industry.

Virulence in avian *E. coli* is a multifactorial characteristic. Adhesion is probably the first step of mucosal colonization and has been associated with the presence of type F1A or type F1A-like pili on avian *E. coli* strains (22,23,24). All isolates belonging to serogroups O1, O2 and O78 produced pili and were able to agglutinate avian red blood cells. It has previously been

reported (25) that pili isolated from serogroup O2 and O78 virulent strains of avian *E. coli* are type F1A or type F1A-like. The failure to observe significant piliation after 18 hours of growth and the mannose-sensitive nature of the hemagglutination provide indirect evidence that the pili observed here were common type 1 (26). While the role of type F1A pili in the pathogenesis of intestinal infections of epithelial surfaces by increased colonization (23,24) has clearly been demonstrated, their role in septicemia remains unclear (8).

A link between virulence and the ability of bacteria to grow under iron-limiting conditions was observed by Dho and Lafont (6). The system of iron uptake mediated by the siderophore aerobactin was observed in 95% of the strains studied. One of the two isolates which did not produce aerobactin was also sensitive to killing by 50% turkey serum. As serum resistance is also associated with pathogenicity, this isolate may not be a pathogen. It will be necessary to test the virulence of the aerobactin negative isolates to confirm their pathogenicity. Other researchers have also found a high frequency of aerobactin production by avian colibacillosis isolates (8,27,28) and demonstrated a correlation with the ability to produce disease.

The production of high molecular weight IROMPS was observed under iron-restricted conditions in all strains tested. In other isolates of *E. coli*, IROMPS of 81 kDa and 74 kDa have been identified as the receptor for ferric enterobactin and aerobactin respectively (16). It is likely that some of the IROMPS identified here are receptors for these siderophores, but further work will be required to confirm the presence of specific receptors. All isolates of serogroup O78 produced the same pattern of IROMPS while considerable variation was observed in the IROMPS produced by serogroup O1 and O2 isolates. This type of variation has been previously reported (16), but the significance of the variation in patterns of IROMPS observed is not known. The production of aerobactin and high molecular weight IROMPS allows the pathogenic *E. coli* strains to grow in low iron conditions encountered during infection of the avian host and therefore contribute to the overall virulence of the bacteria.

Lafont *et al* (29), using tthe one-day-old chick model, classified strains with an LD_{50} of 10^3 to 10^6 CFU as virulent, while those strains with an LD_{50} of 10^8 to 10^{10} CFU were considered avirulent. The organisms with an LD_{50} of 10^6 to 10^8 CFU were classified intermediate in nature. LD_{50} determinations were not done on all strains of serogroups O1, O2 and O78. However, some conclusions can be drawn from the information obtained by challenging day-old chicks with one or two different doses of the individual strains. All *E. coli* strains of serogroups O1 and O2 tested were highly virulent according to this scheme. Three of the five serogroup O78 strains (EC223, EC226, EC343) would be classified as virulent. In contrast, two of the serogroup O78 strains (EC101, EC102) would be considered to be only of intermediate virulence or avirulent as 10^6 CFU of bacteria failed to kill 50% of the birds. Further testing will be necessary to determine accurate LD_{50}'s of these strains.

Serum resistance has been shown to contribute to the virulence of *E. coli* (27,30). All strains of serogroup O1 and O2 were serum resistant. In contrast, the three virulent isolates of serogroup O78 were serum sensitive. This suggests that while serum resistance may play an important role in virulence, other factors must be involved. Polysaccharide capsule type K1 has been shown to inhibit phagocytosis and killing by serum while increasing the pathogenicity of the bacteria (3). The nature of the capsule of the avian isolates described here has not been examined and therefore its contribution to virulence cannot be assessed.

The *E. coli* strains isolated in western Canada from chickens and turkeys with colibacillosis do not appear to differ from those isolated from avian sources in other geographic locations, including Quebec (8). No difference was observed between *E. coli* isolates obtained from turkeys and those obtained from chickens. The collection of *E. coli* isolates described here and those from other areas are comprised of a large number of serogroups and include a significant proportion of isolates that cannot be serotyped. The degree of crossprotection obtained against other serogroups by vaccination with one serogroup is not clear (32,33). In view of the large number of serogroups observed in our strain collection and others, an effective vaccine will need to contain a number of strains of different serogroups. The use of multilocus enzyme electrophoresis to group pathogenic avian *E. coli* into clonal groups may also provide useful information to aid in the selection of suitable vaccine candidates.

ACKNOWLEDGMENTS

We are grateful to the Western College of Veterinary Medicine, University of Saskatchewan, Alberta Agriculture, Animal Health Division, and the Animal Health Center, Ministry of Agriculture and Fisheries, British Columbia for providing the *E. coli* strains described here. Hybrid Turkeys, Kitchener, Ontario kindly provided the turkey poults used.

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