Identification and Purification of F11 Fimbriae on Avian Pathogenic *Escherichia coli*

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Summary

A mannose-resistant hemagglutinating (MRHA) P or P-related fimbriae was purified from three *E.coli* isolates from chickens with colisepticemia. The polyclonal antiserum against the fimbriae was prepared in rabbits. Examination of the purified fimbriae by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated a major fimbriae subunit of approximately 18 kDa. This band was also reacted with anti-F11 serum on Western blotting. The antiserum against the avian P fimbriae strongly reacted with the major subunit of the homologous fimbriae and with F11 fimbriae on Western blotting. The first 21 N-terminal amino acid sequence of the major fimbrial subunit of the avian P fimbriae was identical to that of F11 fimbriae. The adhesive properties of the avian P fimbriae was similar to that of F11 fimbriae with regard to both MRHA of human erythrocytes and binding to the Gal-Gal class II receptor. These results indicate that this fimbriae on avian *E.coli* isolates are closely related to F11 fimbriae which are associated with *E.coli* isolated from human urinary tract infection.

Key words: *Escherichia coli*, F11 fimbriae, chicken, pathogenic, purification

Introduction

Colisepticemia or colibacillosis is one of the principal causes of morbidity and mortality in chickens and turkeys, and is responsible for significant worldwide economic losses (Gross 1991). Bacterial colonization of the respiratory tract is considered to be the initial step in avian colisepticemia and may be mediated by fimbrial adhesions present on the bacterial surface (Klemm 1985). Two main groups of fimbriae, F1 (type 1) and P, have been associated with pathogenic avian *E.coli*. F1

fimbriae, encoded by fim (pil) or related gene clusters are commonly found on both commensal and pathogenic *E.coli* isolates (Dozois *et al* 1995). F1 fimbriae mediate mannose-specific adherence to erythrocytes of numerous animal species, yeast cells, and many other cell types and are termed mannose-sensitive or mannose-sensitive hemagglutinating (MSHA) fimbriae (Duguid & Old 1980). P fimbriae encoded by pap, prs, or related gene clusters (Hacker 1990), are mannose-resistant hemagglutinating fimbriae which have been associated with *E.coli* isolates causing urinary tract infection (UTI) in humans and dogs and septicemia in pigs and poultry (Johnson 1991, Garcia *et al* 1988, Harel *et al* 1993, Ott *et al* 1991, Dozois *et al* 1992, Van den Bosch *et al* 1993). The epithelial receptors for P fimbriae are α-D-galactopyranosyl-β-D-galactopyranoside (Gal-α (4-1) β-Gal) present on the P blood group antigens, but adhesion specificity has been shown to vary among P fimbriae due to differences in the structure of the tip-located G protein adhesions (Dozois *et al* 1995). P and type 1 fimbriae are subject to phase variation, which controls bacterial expression of fimbriae. The *in vitro* environmental conditions controlling phase variation of expression of P fimbriae as well as type 1 fimbriae have been shown to vary among different *E.coli* isolates (Fairbrother *et al* 1988, Dozois *et al* 1995). In one study, 41% of 110 isolates examined from septicemic poultry from Canada, possess DNA sequences hybridizing with *pap* DNA (Dozois *et al* 1992). In other study, P fimbriae of serotype F11 were detected in 78% of 203 avian *E.coli* isolated grown on complex solid medium as determined by immunological methods (Van den Bosch *et al* 1993). Some of *E.coli* isolates in our laboratory from chickens with coliseptisemia expressed P fimbriae *in vitro* as determined by MRHA. Thus, the objectives of the present study were to identify and purify the P fimbriae expressed by *E.coli* isolated from chicken with colibacillosis.

Materials and Methods

Bacterial isolates and culture conditions. Three *E.coli* isolates (582, 583, 683) from the blood and heart of chickens with colisepticemia were used in this study. The isolates are lethal for day-old chicks when inoculated subcutaneously (DL50<103 cfu), and they provoke characteristic lesions of colisepticemia in 3-week old chickens when inoculated into caudal thoracic air sac. Prior to phenotypic and

purification, the isolates were subjected to six consecutive passages of 18h at 37°C on trypticase soy agar (TSA) (Difco Laboratories, Detroit, MI) for maximal production of P fimbriae and minimal production of type 1 fimbriae.

Hemagglutination. MSHA and MRHA tests were performed essentially as described by Fairbrother *et al.* (1988). Isolates were tested for hemagglutination with human OP1 and chicken erythrocytes in the presence or absence of 2.5% D-mannose. For detection of MRHA adhesins specific to P fimbriae, a α-D-galactosyl-(1, 4)-β-galactose (Gal-Gal) latex bead agglutination test was used (Dozois *et al* 1992).

Purification of fimbria. The MRHA-positive isolates were grown on TSA for maximal production of P fimbriae and minimal production of type 1 fimbriae. The isolates were initially passaged six times on TSA at 37°C for 20h to enhance fimbrial production. Bacterial cells were then harvested in Tris buffer (10mM Tris-HCl pH7.4). Fimbriae were removed from the bacteria by heating for 20min at 56°C and by homogenization for 2 periods of 5min on ice in a Sorvall Omnimixer (Omni Corporation International, Waterbury, CT, U.S.A) at half speed. The suspension was centrifuged at 8500rpm for 20min. The pellet was discarded, and the fimbriae were purified from the supernatant by the method of Korhonen *et al* (1980). Briefly, the fimbriae were precipitated overnight at 4°C by adding 20% crystalline ammonium sulfate. After centrigugation at 12,000rpm for 90min, the pellet was suspended in Tris buffer and dialyzed exhaustively against Tris. They were treated with 0.25% sodium deoxycholate (DOC) for 3 days and the DOC insoluble material was removed by centrifugation. They were further purified by ultracentrifugation on a 20 to 60% discontinuous sucrose gradient. After centrifugation, the fimbrial band was recovered and dialyzed against Tris. They were fractionated on a Sepharose-6B gel filtration column (Pharmacia, Uppsala, Sweden). The eluted fractions were concentrated using a stirred ultrafiltration cell (Amicon, W.R. Grace and Co., Danvers, MA, USA.), and examined by TEM and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein estimation. Protein was estimated by the method of Lowry *et al* (1951) using bovine serum albumin as the standard.

Antisera. Polyclonal antisera against the fimbriae from the avian isolate 583 (O1:K) or F11 fimbriae purified from reference strain C1976 (O1:K1:H7:F11) were

prepared in New Zealand white rabbits by intramuscular injection of 200µg of purified fimbriae in Freund's complete adjuvant and then in an equal quantity of incomplete Freund's adjuvant after three weeks according to standard procedures. Six weeks after the initial inoculation, a booster injection of 100µg of fimbriae in Freund's incomplete adjuvant was given intramuscularly. The rabbits were bled 2 weeks after the booster injection. Antisera were absorbed with the homologous strains grown for 2 days at 16°C on TSA to remove non-specific reactions as described (Edwards & Ewing 1972).

SDS-polyacrylamide gel electrophoresis. Discontinuous SDS-PAGE was performed in 1-mm-thick slab gels by a modification of the procedure described by Laemmli (1970). Stacking and separating acrylamide gels of 5.0% (0.125M Tris hydrochloride [pH6.8]) and 15% (0.375M Tris hydrochloride [pH8.8]), respectively, were prepared in mini-gel apparatus (Paya Pajoohesh Co., Mashhad, Iran). Electrophoresis buffer was 25mM Tris hydrochloride-0.2M Glycine solution (pH8.3) with 0.1% SDS. Samples were diluted 1:1 in 60mM Tris hydrochloride buffer with 2% SDS-10% Glycerol-0.002% bromophenol blue-5% a-mercaptoethanol and boiled for 10min. Electrophoresis was carried out at a constant voltage of 100 for about 3h. Prestained low-molecular-weight markers (Bio-Rad) were used as standards. Gels were stained with Coomassie brilliant blue R250 and examined for the presence of lipopolysaccharide (LPS) contaminants by silver staining (Hitchccock & Brown 1983).

Western blotting. After SDS-PAGE, separated bacterial components were transferred to nitrocellulose membranes by electrophoretic blotting (Uniform Electro Transfer, Paya Pajoohesh Co., Mashhad, Iran) at 100V for 2h in 20mM Tris, 150mM Glycine, and 20% methanol (pH8.6). The nitrocellulose membranes were then incubated serially in the following solutions (I) a 0.5% (w/v) dilution of bovine serum albumin, (ii) a suitable dilution of rabbit antiserum raised against the appropriate fimbrial antigen, (iii) a 1:5000 dilution of goat anti-rabbit IgG with peroxidase (Bio-Rad). Each incubation was carried out at room temperature for 2h and followed by a 5min wash in 0.02M Tris-HCl (pH7.5) containing 0.5M NaCl and 0.2% (v/v) Tween-20 and a 5min wash in 0.02M Tris-HCl (pH7.5) containing 0.5M NaCl. The membranes were then incubated at room temperature for 15min in a color

development solution containing 4-chloro-1-naphthol prepared according to the manufacturer's instructions (Bio-Rad).

Transmission electron microscopy (TEM). Bacterial cells and purified fimbriae was examined by transmission electron microscopy after negative staining with 2% phosphotungstic acid as previously described with slight modifications (Fairbrother *et al* (1986). Briefly, a drop of purified fimbriae was placed on a 200-mesh Formvar-coated carbon electron microscope grid and then allowed to air dry. The grids were then floated on a drop of 2% (w/v) phosphotungstic acid (pH7.2). After 1min, excess liquid was removed by touching the edge of the grids with a piece of filter paper. The grids were then allowed to air dry. Grids were examined on a Philips 400 electron microscope operating at 100-kV, 50-nA emission.

N-terminal sequence analysis. The avian P purified fimbriae was electroblotted onto Immobilon-P transfer membrane using CAPS buffer (pH11.0). Automated Edman degradation was performed on an Applied Biosystem Gas-Phase Sequencer (Model 470A) equipped with an on-line phenylthiohydantoin analyzer (Model 120A, Applied Biosystems) using the general protocol of Hewick *et al* (1981). Samples were applied to the TFA-treated cartridge filters, coated with 1.5mg of polybrene, and 0.1mg of NaCl (Biobrene Plus, Applied Biosystems). A standard program (03RPTH) was employed for sequencing. The phenylthiohydatoin amino acid derivatives were determined by comparison with standards (PTH Analyser Standards, Applied Biosystems) and analyzed on-line prior to sequence analysis.

Results

Three isolates showed MRHA of human OP1 erythrocytes and specific Gal-Gal binding typical of P adhesions. MRHA was not observed when the bacteria were grown on solid medium at 16°C. On the other hand, the isolates which were MRHA positive, were usually fimbriated when grown on solid media at 37°C but were nonfimbriated or poorly fimbriated when grown at 16°C in solid media (Figure 1). P fimbriae was well expressed after six passages of bacteria on TSA and production of type 1 fimbriae was absent. Fimbriated isolate hemagglutinated human and chicken erythrocyes in the presence of mannose. MRHA-positive isolate 583 (O1:K1) was chosen for fimbrial purification. The fimbriae on the MRHA-positive bacteria grown

on TSA were long and straight and had a diameter of approximately 8nm (Figure 2). The average yield of F11 fimbriae from 10 liters cultures was 1.7mg. The level of purity was very high after gel filtration.

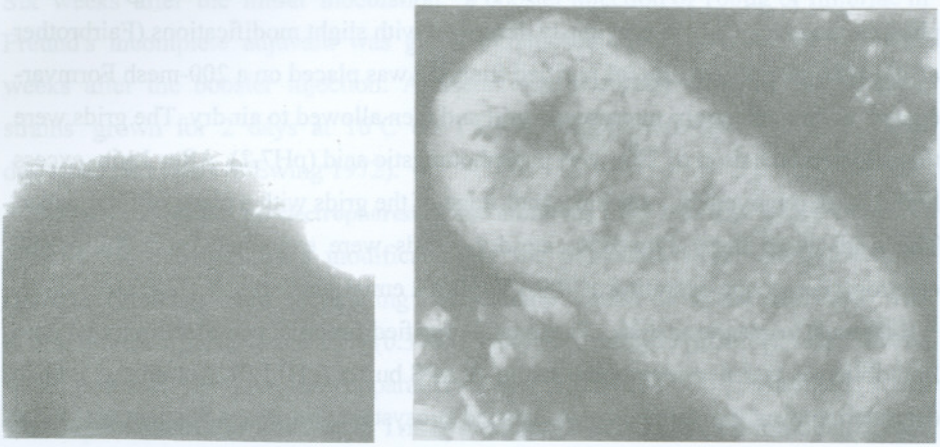

Figure 1. *Electron micrograph of E. coli 583 grown on TSA at 37°C stained with 2% phosphotungstic acid showing long, thin fimbriae attached to the surface of the cell. X70,000. (left). Electron micrograph of E. coli 583 grown on TSA at 16°C stained with 2% phosphotungstic acid. Cell was nonfimbriated when grown on TSA at 16°C. X60,000 (right).*

Figure 2. *Fimbriae isolated from E. coli 583 negatively stained with 2% phosphotungstic acid. The fimbriae had a diameter of approximately 8nm. X125,000.*

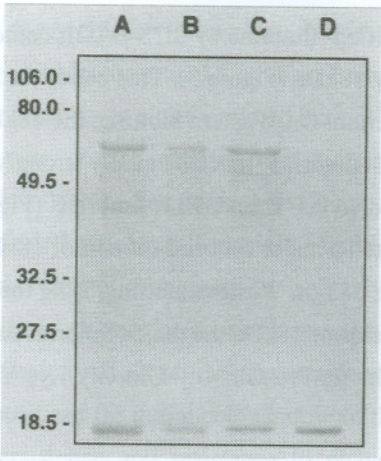

Figure 3. *SDS-polyacrylamide gel electrophoresis of preparations at different steps of purification of fimbriae from isolate 583. (A) the crude fimbrial preparation after mechanical shearing and precipitation with ammonium sulfate; (B) the fimbrial preparation after dialysis against Tris buffer and treatment with sodium deoxycholate; (C) the deoxycholate soluble fraction after centrifugation; (D) the purified fimbriae after gel filtration. The apparent molecular weights of the marker protein (x 10^3) are indicated on the left.*

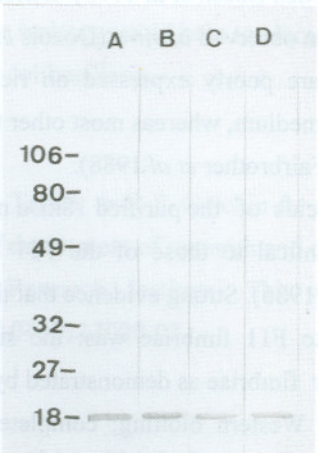

Figure 4. *Western blotting of purified fimbriae with antiserum against the avian P fimbriae of isolate 583 (A, B) and F11 fimbriae (C, D). (A, C) purified F11 fimbriae; (B, D) purified fimbriae from avian isolate 583. The apparent molecular weights of the marker protein (x 10^3) are indicated on the left.*

Examination of the purified fimbriae by SDS-PAGE demonstrated a major fimbrial subunit of approximately 18kDa (Figure 3). This band was also observed on Western blotting using anti-F11 serum. On Western blotting, the 18kDa major fimbrial subunit of the avian P and F11 fimbriae reacted equally strongly with the absorbed serum against both the purified avian P and F11 fimbriae (Figure 4). Two other *E.coli* isolates also demonstrated a major fimbrial subunit of 18kDa, which reacted equally strongly to that of isolate 583 on Western blotting using the anti-F11 serum. The first 21 N-terminal amino acids of 18kDa the major fimbrial subunit of avian strain were identified as Ala-Pro-Thr-Ile-Pro-Gln-Gly-Gln-Gly-Lys-Val-Thr-Phe-Asn-Gly-Thr-Val-Val-Asp-Ala-Pro.

Discussion

In this study, we have demonstrated that pathogenic *E.coli* isolated from chickens with colisepticemia expressed P fimbriae *in vitro* when grown on solid media at 37°C. Phase variation of expression of P fimbriae may be enhanced or inhibited by different environmental conditions. MRHA fimbriae such as P fimbriae are generally well expressed on agar (van den Bosch *et al* 1993). Differences in expression among P fimbriae types have been observed *in vitro* (Dozois *et al* 1995). For instance, the Prs-like $F165_1$ fimbriae are poorly expressed on rich solid medium, but well expressed on minimal solid medium, whereas most other types of P fimbriae are well expressed on rich medium (Fairbrother *et al* 1988).

The N-terminal amino acids of the purified 18kDa major fimbriae subunit from the avian strains were identical to those of the F11 major fimbriae subunit as published by Van Die *et al* (1986). Strong evidence that this fimbriae on avian *E.coli* isolates is closely related to F11 fimbriae was: the similar molecular size of the major subunits of the their fimbriae as demonstrated by migration on SDS-PAGE; complete cross-reaction on Western blotting; complete identify of the first 21 N-terminal amino acids; identical adhesive properties with regard to both MRHA of human erythrocytes and binding to the Gal-Gal class II receptor for three avian isolates (Dozois *et al* 1995). Similarly, van den Bosch *et al* (1993) found that P fimbriae produced by pathogenic avian *E.coli* were identical to F11 as demonstrated by Western blotting, ELISA, and N-terminal amino acid analysis. They found that

the most *E.coli* isolates from chickens were positive for F11 as demonstrated by ELISA. In previous study, we demonstrated that avian P fimbriae from *E.coli* strains isolated from septicemic turkeys is closely related but not identical to F11 fimbriae which are associated with *E.coli* isolated from human urinary tract infection (Pourbakhsh & Fairbrother 1994). The high prevalence of F11 expression among strains isolated from infected chickens, especially among strains with the most prevalent O:K serotypes, strongly suggests a role for F11 fimbriae in the pathogenesis of colibacillosis. It has been previously demonstrated that P fimbriae are important for pathogenicity of *E.coli* causing septicemia in pigs experimentally inoculated by the oral route, not for initial colonization of the intestinal mucosa, but for systemic bacterial persistence and resistance to phagocytosis (Ngeleka *et al* 1994). *In vivo* expression of P fimbriae in chickens experimentally inoculated with pathogenic *E.coli* isolates, which express the fimbriae in *in vitro* condition, has been demonstrated. P fimbriae were expressed *in vivo* by bacteria colonizing air sacs, lungs, kidney, blood, and pericardial fluid of chickens suggesting that P fimbriae may be involved in the colonization of systemic organs and in the development of septicemia (Pourbakhsh *et al* 1997). Infection studies using isogenic mutants in the *pap* fimbrial system of these isolates would help to elucidate the role of this fimbriae in the pathogenesis of avian colibacillosis.

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