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Characterization of fimbriae extracts from porcine enterotoxigenic *Escherichia coli* strains carrying F6 (987P) antigen

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Summary. Fimbrial extracts from porcine enterotoxigenic *Escherichia coli* (ETEC) strains carrying F6 (987P) intestinal colonization factor antigen were obtained using the thermal shock method. The extracts were analyzed by SDS-PAGE and immunoblotting using different fimbriae-specific antisera. Two major protein bands with molecular masses of 17.5 and 21.9 kDa were detected. The 21.9-kDa band was identified as the major subunit of F6 fimbrial antigen in strains of serogroups O9 and O141. The 17.5-kDa band was associated with porcine strains of serogroups O9 and O20. [**Int Microbiol** 2006; 9(4):241-246]

Key words: *Escherichia coli* · enterotoxigenic *E. coli* · F6 antigen · 987P antigen · enterotoxins · porcine diarrhea · immunoblotting

Introduction

Neonatal diarrhea is a common major infectious disease of swine, causing high mortality rates in piglets [9,21]. This disease is associated with a small number of serotypes of enterotoxigenic *Escherichia coli* (ETEC) strains [2,10]. These non-invasive bacteria cause diarrhea by secreting heat-labile (LT) and heat-stable (STa and STb) enterotoxins that interact with the intestine to yield the electrolyte-rich, watery diarrhea characteristic of the illness [12]. A major factor influencing ETEC pathogenicity is the close adherence of the bacteria to the intestinal wall, which is mediated by fimbriae. Epidemiological studies have shown that ETEC isolates from piglets with diarrhea may express F4 (K88), F5 (K99), F6 (987P), F18, and F41 antigens or additional fimbrial adhesions (colonization factors) whose structures are not yet completely determined [2–4,11,14,27,28]. Fimbriae consist of one major structural subunit assembled in a helical fashion and arranged over the surface of the bacterium. In addition, most fimbriae incorporate subunits as minor components [7]. The fimbrial proteins are efficiently expressed when the bacteria are grown at 37ºC (but not at 18ºC), allowing specific sera against fimbriae to be obtained. Adhesion to the mucosa is mediated by glycoprotein and glycolipid receptors on the brush borders of porcine intestinal cells [18,29]. Parenteral vaccination of pregnant sows with strains expressing the specific fimbriae conferred protection to their suckling piglets against infection with homologous strains expressing the same fimbriae [20,26]. The importance of adhesins in the

pathogenicity of ETEC resulted in studies on their usefulness in vaccines. Identifying which fimbrial variant occurs most frequently in the ETEC strains isolated from a particular geographical region is crucial to the development of more effective vaccines to prevent ETEC diarrhea in piglets [26]. The aim of the present study was to characterize the fimbrial extracts of porcine ETEC strains carrying F6 (987P) antigen.

Materials and methods

Bacterial strains. ETEC strain 987 served as the control strain for F6 antigen and was previously described by Moon [20]. Two additional F6 control strains (H364/83 and H243/84) were supplied by Jansen and Guinée (National Institute of Public Health and Envirommental Protection, Bilthoven, The Netherlands). Spanish porcine strains (Ip102 and Ip19a) were used as controls for F4, F5, and F41 colonization antigens. The characteristics of these five ETEC control strains and 17 other ETEC strains carrying F6 antigen that were isolated in Spain from piglets with diarrhea [2,10,11] are listed in Table 1. Strains were conserved in tryptone soy broth (TSB) containing 30% v/v glycerol at –30ºC.

Detection of virulence genes by PCR and toxin production. All 22 ETEC strains were tested with primers specific for the genes encoding toxins (LT, STa, STb, VT1, VT2, and VT2e) and adhesins (F4, F5, F6, F18, and F41) as described elsewhere [1,3]. In addition, the infant mouse test was used to test strains for the production of STa enterotoxin; LT enterotoxin and verotoxin production were assayed on Vero cells as previously described [2].

Serotyping. The presence of O antigen was determined as previously described [10] and employing all available O (O1–O185) antisera. O antisera were produced in the *E. coli* Reference Laboratory (LREC) at the University of Santiago de Compostela, Lugo, Spain, and absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins.

Growth conditions for fimbrial expression. The phenotypic expression of fimbriae was determined by growing the bacteria in Mueller Hinton broth (Difco). The cultures were incubated at 37ºC for 5 days under static conditions until a definite pellicle had formed. Bacteria obtained from the pellicle were inoculated onto colonization factor agar (CFA) [8] (for F4 and F6 expression) or Minca Isovitale X agar [14] plates (for F5 and F41 expression) and incubated at 37ºC for 18 h. Fimbrial antigen expression by the bacterial strains was determined using the staphylococci coagglutination test with specific polyclonal antisera against F4, F5, F6, and F41 fimbrial antigens.

Extraction of fimbriae. Bacteria from five plates of CFA agar were harvested in 20 ml of 50 mM phosphate buffer, pH 7.2, containing 2M urea. The bacterial suspension was heated at 60ºC for 30 min to remove fimbriae, followed by centrifugation (6000 ×*g* for 30 min). Fimbriae were precipitated using ammonium sulfate (60% saturation) and then centrifuged at 6000 ×*g* for 1 h. The precipitate was dissolved in 5 ml of the same buffer used for extraction and then dialyzed against 50 mM phosphate buffer, pH 7.2.

Antisera production. Six hyperimmune antisera specific against fimbrial antigens were obtained from rabbits immunized with whole bacterial cells expressing F6 antigen. The rabbits received multiple intravenous injections of bacteria that were formalin-killed for the first four injections and alive for the last two: antiserum 60 (Pd25d/O9/17.5 kDa), antiserum 62 (Pd23a/O9/17.5 kDa), antiserum 7 (Pd26c/O9/21.9 kDa and 17.5 kDa), antiserum 32 (Pd114b/O9/21.9 kDa), and antisera 42 and 49 (Ip49a/O141/21.9 kDa). To remove any non-specific antibodies, the antiserum was extensively adsorbed with suspensions of homologous bacteria heated at 121ºC for 90 min and homologous live bacteria grown at 18ºC that did not express fimbrial antigen.

Four specific antisera against major fimbrial subunits were obtained using pieces of gel containing the major subunit after determining the position of the protein band by Coomassie staining: antiserum B1 (Pd25d/O9/17.5 kDa), antiserum B2 (Pd23a/O9/17.5 kDa), antiserum B3 (Pd114b/O9/21.9 kDa), and antiserum B4 (Ip84a/ O141/21.9 kDa). The gel pieces were homogenized with 1 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.9% NaCl. Antisera were obtained following four subcutaneous injections at 2-week intervals. The initial two injections were given in Freund's complete adjuvant containing 500 µl of suspension, and the two subsequent injections in Freund's incomplete adjuvant containing 250 µl of suspension. The final bleeding was done 15 days after the last immunization.

Table 1. Characteristics of enterotoxigenic *Escherichia coli (*ETEC) strains and of the major subunits detected in bacterial heat extracts after SDS-PAGE

Strain	Serogroup	Enterotoxins	Colonization antigen	Major subunit (kDa)
Ip102	O149	LT, STa, STb	F4 (K88ac)	29.2
Ip19a	09	STa	F5 and F41	30.9 and 17
987	O9	STa, STb	F6	21.9
H364/83	O20	STa	F6	17.5
H243/84	O9	STa	F6	21.9
Pd22b	O9	STa	F6	21.9 and 17.5
P23a	O9	STa	F6	17.5
Pd25d	O9	STa	F6	17.5
Pd26d	O9	STa	F6	21.9 and 17.5
Pd26c	O9	STa	F6	21.9 and 17.5
Pd114b	O9	STa	F6	21.9
Pc68b	O9	STa	F6	21.9 and 17.5
Ip42c	O141	STa, STb	F6	21.9
Ip49a	O141	STa	F6	21.9
Ip51b	O141	STa	F6	21.9
Ip81d	O141	STa	F6	21.9
Ip84a	O141	STa	F6	21.9
Ip85b	O141	STa	F6	21.9
Ip89a	O141	STa	F6	21.9
Ip89c	O141	STa	F6	21.9
Ip92b	O141	STa	F6	21.9
Ip92c	O141	STa	F6	21.9

SDS-PAGE and immunoblotting. SDS-PAGE was carried out following the method of Laemmli [19] using the Protean-II system (BioRad, USA). Immunoblotting was done as described by Towbin et al. [24] with minor modifications, as previously described [13, 25].

Dot-blot assay. Bacteria expressing fimbrial antigens were suspended in 50 mM phosphate buffer, pH 7.2, and the concentration was adjusted to A_{540} = 1.8. The bacteria were transferred onto nitrocellulose strips using a vacuum system (Gybco BRC, Filtration Manifold System). The membrane was removed, blocked with 3% BSA in TBS-T (10 mM Tris-HCl, pH 7.4, containing 0.05% (v/v) Tween-20) at 37ºC for 1 h, immersed in specific sera diluted 1/1000 in TBS-T, and incubated at room temperature for 1 h with gentle agitation. The membrane strips were then washed and fimbrial antigens were detected using goat anti-mouse IgG horseradish-peroxidase conjugate, as described earlier [13].

Electron microscopy. Bacterial cells were suspended in PBS, applied to carbon-coated nickel grids, and negatively stained with 2% ammonium molybdate. The grids were examined in a JEOL JEM-100 SX at 60 kV.

Results

Virulence genes. All 22 strains had genes encoding STa enterotoxin: F6 adhesin (20), STb enterotoxin (3). LT enterotoxin (1), F4 (1), F5 (1), and F41 (1). All of these strains were negative for genes encoding VT1, VT2, and VT2e verotoxins and F18 adhesin.

SDS-PAGE and immunoblotting. Electrophoretic separation of F6 antigen from the heated fimbrial extracts of 20 strains indicated in Table 1 showed two major proteins, with molecular masses of 17.5 and 21.9 kDa (Fig. 1). These two protein bands were detected neither in heat extracts from strains grown at 18ºC nor in control strains that did not express F6 antigen. Each strain was heat-extracted three times. A 17.5-kDa protein was consistently obtained in all of the extracts from the strain expressing this protein. By contrast, the 21.9-kDa band was not always present in all three extracts from the same strain.

The 17.5- and 21.9-kDa proteins reacted with the adsorbed antisera against F6 antigen obtained from the various strains: antiserum 42, obtained against strain Ip49 (which showed only the 21.9-kDa protein), reacted with the 21.9-kDa protein; antiserum 60, obtained against strain Pd25d (which showed only the 17.5-kDa protein), reacted with the 17.5-kDa protein; and antiserum 7, obtained against strain Pd26c (which showed the 21.9- and 17.5-kDa proteins), reacted with both protein bands. Several proteins with molecular masses ranging between 49.5 and 32.5 kDa also reacted with adsorbed polyclonal antiserum, but the reactions were not consistent (Fig. 2).

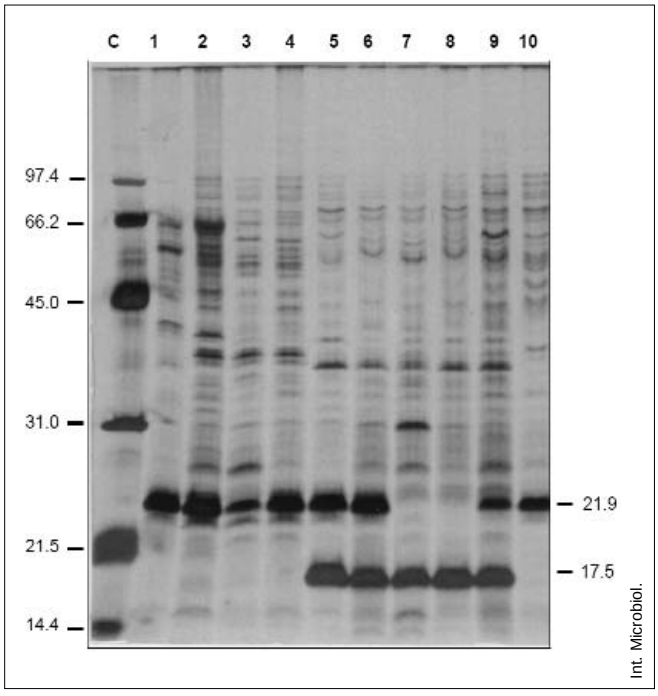

Fig. 1. SDS-PAGE of F6 fimbriae after heat extraction and ammonium sulfate (60% saturation) precipitation. C: marker proteins. Strains: lane 1, Pd114b; lane 2, Ip85b; lane 3, Ip84a; lane 4, Ip 42c; lane 5, Pc68b; lane 6, Pd26d; lane 7, Pd25d; lane 8, Pd23a; lane 9, Pd22b; lane 10, 987.

Specific antisera against the 21.9- and 17.5-kDa protein bands were obtained. Antisera B3 and B4, produced against the 21.9-kDa protein, reacted with this protein and with a protein of approximately 35 kDa. Antisera B1 and B2, produced against the 17.5-kDa protein, reacted additionally with two proteins with molecular masses ranging from 35 to 42 kDa. Reaction with the proteins of higher molecular mass was not consistent and depended on the extract used (Fig. 2). The 21.9-kDa protein was identified in the control strain 987, in which F6 antigen was discovered. The molecular mass of this protein was affected by the presence or absence of b-mercaptoethanol in the SDS-PAGE buffer. The 17.5-kDa protein was not affected by this treatment.

Dot-blot assay. Antisera against bacteria whose heat extracts contained the 17.5-kDa protein (antisera 60, 62, and 7) only reacted with strains containing this protein in their heat extracts. Similar results were observed with antisera against bacteria containing the 21.9-kDa protein in their extracts (antisera 7, 42, 49, and 32). Antisera against the 21.9-kDa protein that was obtained by SDS-PAGE (antisera B3 and B4) did not react with any strain in the dot-blot assay. By contrast, antisera obtained against the 17.5-kDa protein from SDS-PAGE (antisera B1 and B2) reacted with all of the strains that had this protein in their heat extracts. These latter antisera did not react with strains expressing mannose-sensitive hemagglutinin (MSHA) (pilus type 1), F4, F5, and F41 antigen, and the reaction persisted after the antiserum had been adsorbed with strains whose heat extracts contained only the 21.9-kDa protein.

Electron microscopy. Negative-staining electron microscopy of strains 987, Ip49a, and Pd 25d detected thin, long, morphologically similar fimbriae in all of the strains. Thin fimbriae were frequently aggregated (Fig. 3).

Discussion

F6 antigen was first described by Isaacson and Richter [15] as a long, straight structure surrounding bacteria. These authors reported a molecular mass of 20.6 kDa for the major subunit, which decreased to 19.5 kDa when β-mercap-

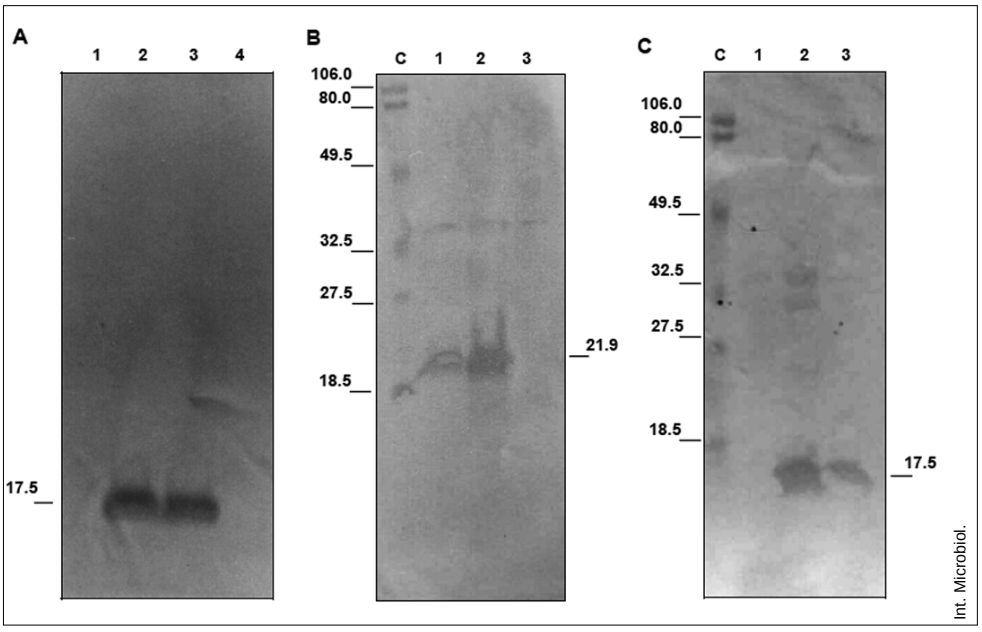

Fig. 2. Immunoblotting of fimbrial suspensions. (**A**) Immunoblots developed with antiserum number 60. Strains: lane 1, Ip42c; lane 2, Pd25d; lane 3, Pd23a; lane 4, Ip92c. (**B**) Immunoblots developed with antiserum B3. Strains: lane C, marker proteins; lane 1, 987; lane 2, Pd22b; lane 3, H364/83. (**C**) Immunoblots developed with antiserum B1. Strains: lane C, marker proteins; lane 1, 987; lane 2, Pd22b; lane 3, H364/83.

toethanol was eliminated from the buffer. De Graaf and Klaasen [6] reported that five proteins with molecular masses of 81.0, 39.0, 28.5, 20.5, and 16.5 kDa are necessary for the expression of this antigen. The 20.5-kDa protein was identified as the major subunit. Schifferli et al. [23] suggested that these proteins had different roles and that adhesion to intestinal epithelial cells is closely associated with the expression of fimbriae. F6 fimbriae are composed of multiple copies of one fimbrial protein subunit, FasA, and two minor subunits, FasF and FasG, arranged along a filamentous axis [5,17]. FasG was described to be essential for specific adhesion to receptors in the intestine [18]. Schifferli et al. [23] also found that antibodies against F6 antigen did not recognize minor components of the subunit. We have demonstrated that polyclonal antibodies obtained against strains expressing F6 fimbriae reacted with two proteins, with molecular masses of17.5 and 21.9 kDa, in a strain-specific manner. Additional reactions with proteins with molecular masses between 35 and 42 kDa were also observed. Neither the 17.5- nor the 21.9-kDa protein was expressed at 18ºC. The inconsistent expression of the 21.9-kDa protein, together with its behavior in the absence of b-mercaptoethanol in the buffer, suggested that this protein correlates with the one described by Isaacson and Richter [15]. The results of experiments using antibodies obtained against the two protein bands isolated directly from polyacrylamide gels showed that these proteins are immunologically different; the reaction of some sera with both proteins was due to the common expression of both structures by these strains.

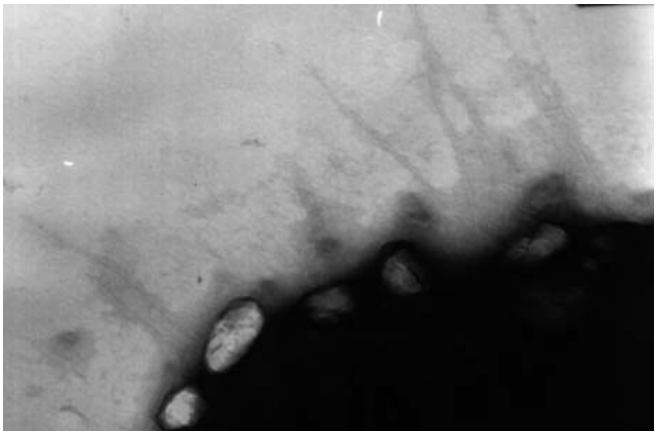

Fig. 3. Negative-staining electron microscopy of strain Pd25d, which showed only the 17.5-kDa protein.

Other authors have also reported reactions obtained against proteins with higher molecular masses [4]. Kennan and Monckton [16] reported the reaction of a 53-kDa protein with an antiserum obtained against the major 17-kDa subunit of Av24 fimbriae. These results, together with ours, suggest that such reactions correspond either to dimers or trimers of the major subunit or to different conformational structures of the same protein. However, it cannot be ruled out that these proteins represent other fimbrial components, such as FasB, which partially migrate through the bacterial membrane together with the major subunits. It may also be the case that since these proteins are not expressed at 18ºC they might not be eliminated in the adsorption process to obtain specific antisera against fimbrial antigens.

Antibodies against the 21.9-kDa protein obtained from polyacrialmide gel did not react with complete cells. This result is in agreement with those obtained by Schifferli et al. [22], in which adhesion to intestinal cells could only be blocked with antibodies obtained against native structures. These antibodies reacted against native structure, whereas antibodies that reacted against the subunits did not always react with native structure of the protein.

The fimbriae of strains expressing the 17.5-kDa protein are similar to those of strains that express the 21.9-kDa band. We observed the 17.5-kDa protein in strains of serogroups O9 and O20. Our results therefore suggest the presence of a new fimbrial antigen that is associated with F6 antigen.

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Caracterización de los extractos de fimbrias de cepas porcinas de *Escherichia coli* enterotoxigénica con el antígeno F6 (987P)

Resumen. Los extractos de fimbrias obtenidos usando el método de choque térmico a partir de cepas de origen porcino de *Escherichia coli* (ETEC) enterotoxigénica con el antígeno de colonización intestinal F6 (987P) fueron analizados por SDS-PAGE e immunotransferencia usando diferentes antisueros específicos frente a antígenos de fimbrias. Se detectaron dos bandas principales de proteínas de 17,5 kDa y 21,9 kDa según la cepa ensayada. La banda de 21,9 kDa fue identificada como la subunidad estructural principal del antígeno de fimbria F6 y se observó en las cepas de los serogrupos O9 y O141. La banda de 17,5 kDa se asoció a las cepas porcinas de los serogrupos O9 y O20. [**Int Microbiol** 2006; 9(4):241-246]

Palabras clave: *Escherichia coli* · *E. coli* enterotoxigénica · antígeno F6 · antígeno 987P · enterotoxinas · diarrea porcina · inmunotransferencia

Caracterização dos extratos de fímbrias de cepas suínas de *Escherichia coli* enterotoxigénica com o antígeno F6 (987P)

Resumo. Os extratos de fímbrias obtidos usando o método de choque térmico a partir de cepas de origem suíno de *Escherichia coli* (ETEC) enterotoxigénica com o antígeno de colonização intestinal F6 (987P) foram analisados por SDS-PAGE e immunotransferencia usando diferentes antisoros específicos para antígenos de fímbrias. Duas bandas majoritárias de proteínas de 17,5 kDa e 21,9 kDa foram detectadas dependendo da cepa testada. A banda de 21,9 kDa foi identificada como a subunidad estrutural principal do antígeno de fímbria F6 e se observou nas cepas dos sorogrupos O9 e O141. A banda de 17,5 kDa se associou com as cepas suínas dos sorogrupos O9 e O20. [**Int Microbiol** 2006; 9(4):241-246]

Palavras chave: *Escherichia coli* · *E. coli* enterotoxigénica · antígeno F6 · antígeno 987P · enterotoxinas · diarréia suína · immunotransferência