Pathotypes and serogroups of enterotoxigenic *Escherichia coli* isolated from pre-weaning pigs in north Vietnam

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The contribution of enterotoxigenic *Escherichia coli* (ETEC) to pre-weaning diarrhea was investigated over a 6 month period at five selected commercial piggeries (CPs) in north Vietnam with at least 100 sows each. Diarrhoea was found to affect 71.5% of the litters born during the period of study. Of 406 faecal specimens submitted for bacteriological culture, 200 (49.3%) yielded a heavy pure culture of *E. coli* and 126 (31%) were confirmed by PCR to carry at least one of eight porcine ETEC virulence genes. ETEC was responsible for 43% of cases of diarrhoea in neonatal pigs during the first 4 days of life and 23.9% of the remaining cases up until the age of weaning. Pathotypes were determined by PCR for the 126 ETEC isolates together with 44 ETEC isolates obtained from village pigs (VPs) raised by smallholder farmers. The CP isolates belonged to five pathotypes, four of which were also identified in VP isolates. Haemolytic serogroup O149:K91 isolates that belonged to F4/STa/STb/LT were most commonly identified in both CPs (33% of isolates) and VPs (45.5%). Other combinations identified in both production systems included O64 (F5/STa), O101 (F4/STa/STb) and O-nontypable (F~/STb). A high proportion of CP isolates (22.3%) possessed all three enterotoxins (STa/STb/LT), lacked the genes for all five tested fimbriae (F4, F5, F6, F41 and F18) and belonged to serogroup O8. These unusual O8 F~ isolates were haemolytic and were isolated from all ages of diarrhoeic piglets at each CP, suggesting that they have pathogenic potential.

**INTRODUCTION**

Vietnam is a developing country with a rapidly expanding commercial pig industry. Whilst the majority of production still relies heavily upon smallholding farmers raising small numbers (<20 sows) of indigenous breeds, the number of large-scale intensive piggeries (>100 sows) utilizing imported or cross-bred stock has increased rapidly over the last 10 years (Hoang, 2003). Increased intensification of the industry has resulted in a substantial rise in health-related problems. Whilst respiratory and enteric diseases in growing pigs are recognized, pre-weaning diarrhoea is also a significant problem (Cu et al., 1999; Nguyen, 1995; Trinh, 1980).

In other major pig-producing countries, enterotoxigenic *Escherichia coli* (ETEC) is the most significant pathogen during the suckling period. Two syndromes are usually recognized: neonatal diarrhoea (ND) in the first 4–5 days of life and scours at 2–3 weeks of age (Bertschinger & Fairbrother, 1999; Fairbrother, 1999a). In suckling pigs, ETEC strains attach to the intestinal epithelium through the action of F4, F5, F6 or F41 fimbrial adhesins and liberate heat-stable (STa, STb) and/or heat-labile (LT) enterotoxins, causing hypersecretory diarrhoea (Nagy & Fekete, 1999; Nataro & Kaper, 1998). ND caused by ETEC is usually controlled by vaccination of the sow in the weeks preceding parturition to ensure that protective maternal antibodies are transferred to newborn pigs through the colostrum. In large commercial piggeries (CPs) in Vietnam, reports of vaccination failure are widespread using both a locally produced killed whole-cell vaccine containing F4 antigen and an...
imported vaccine incorporating F4, F5, F6 and F41 antigens. Furthermore, smallholder farmers in Vietnam and some of the larger CPs do not routinely practice prophylactic vaccination of sows for the control of ETEC (Phu H. Cu, personal communication).

As far as we are aware, there have been no previously published studies conducted in Vietnam to determine the significance of ETEC as a cause of pre-weaning diarrhoea. In addition, very little information is available on which ETEC pathotypes and serogroups are currently present in Vietnamese piggeries. Diagnosis of E. coli as causative agents of diarrhoea in Vietnam has mainly been based on clinical signs, the age of affected pigs and the isolation of a pure, heavy growth of E. coli from faecal specimens submitted to a veterinary diagnostic laboratory. PCR amplification of ETEC fimbriae and enterotoxin virulence genes is now used routinely for ETEC identification in other pig-producing countries (Blanco et al., 1997; Ngeleka et al., 2003; Ojeniyi et al., 1994; Osek et al., 1999). These methods are not currently used in veterinary diagnostic laboratories in Vietnam.

The present study was conducted to provide baseline information on the significance of specific ETEC serogroups and pathotypes associated with pre-weaning diarrhoea in piglets in north Vietnam. The major objectives were to determine the significance of ETEC as a cause of pre-weaning diarrhoea in five selected large CPs, identify the major ETEC serogroups and pathotypes present in the CPs and compare them to a collection of ETEC isolates obtained from VPs raised by smallholding farmers.

METHODS

Participating commercial piggeries. For this study, it was not possible to randomly select participant piggeries due to the diversity of management and husbandry practices existing within north Vietnam. One piggery was instead chosen from each of the five provinces covering the Red River Delta, the midlands and coastal areas according to the following selection criteria: (i) the piggery had a recognized scouring problem in pre-weaning piglets and was not using vaccines for the control of ETEC; (ii) the mean number of sows was ≥100; (iii) the piggery was close enough to the National Institute of Veterinary Research (NIVR), in Hanoi, so that specimens could be processed on the day of collection; and (iv) the piggery’s owner and staff were willing to participate in the study.

Case definitions and data management. A template for data recording was designed and introduced to the staff of each piggery. The following data were recorded for each sow farrowing between January and June 2001: sow parity, number of liveborn piglets, age of each piglet when diarrhoea was first observed and the number of piglets with diarrhoea in the litter. Data were recorded for each sow until the piglets were weaned at 21 days of age. A case of diarrhoea was recorded if a suckling piglet was observed to pass liquid, watery faeces and showed signs of dehydration between birth and weaning. A litter was recorded as being affected by diarrhoea when more than 25 % of piglets in the litter were scouring.

Specimen collection, transport and storage. Faecal specimens were only obtained from piglets with diarrhoea. Only one specimen was obtained for bacteriological investigation from a single representative piglet from each litter with diarrhoea. All specimens were obtained within 24 h of the onset of illness and before any antimicrobial treatment had begun to ensure optimum recovery of E. coli. All specimens were processed on the day of collection. In some cases, specimens were obtained following post-mortem examination of moribund animals during monthly farm visits. In these cases, a sample of the intestinal contents from the ileum was collected for bacteriological examination.

Bacteriology. Specimens were plated in parallel onto sheep blood agar (SBA) and MacConkey agar (MCA). The plates were incubated aerobically overnight at 37 °C and then examined for the presence of bacterial colonies. The presence or absence of haemolytic activity surrounding the colonies growing on SBA was also noted. Isolates of E. coli (originating from a single representative colony on the SBA plate) were considered to be significant only if heavy, pure or nearly pure growth was observed on both SBA and MCA plates. Isolates obtained from the five CPs during the 6 month study were compared with a collection of 62 isolates obtained from VP sources in north Vietnam held at the NIVR. All isolates were confirmed to be E. coli using standard biochemical procedures (indole, methyl red, Voges–Proskauer and citrate utilization tests). After isolation, the bacteria were stored in serum agar stabs at room temperature or in brain heart infusion broth containing 20 % glycerol at −70 °C. Isolates were not subcultured more than twice before being examined by PCR for the presence of ETEC virulence genes. An E. coli isolate was classified as a strain of ETEC if it carried any of the three enterotoxin virulence genes detected by PCR assay.

PCR for determination of ETEC virulence genes. E. coli reference strains (Do et al., 2005; Osek, 1999) were used as positive controls for each virulence factor detected by multiplex PCR, and a non-pathogenic commensal E. coli strain was used as a negative control. Strains 1500 (F6), 1547 (O9:F5), 1520 (O101:F41), 214:081 (O139:K12:H1, F18) and C600 (K-12) were kindly provided by Dr Jacke Osek, National Veterinary Research Institute (PIWet), Poland. Strains FV847a (O149:F4, LT StA STb) and FV2586 (O157:F4, LT STb) were kindly provided by Professor Dr Jorge Blanco Alvarez (Laboratorio de Referencia de E. coli, Spain). Strain 1078/01 (O149:K91:F4) was provided by the National E. coli Reference and Serotyping Laboratory, Department of Primary Industries, Epsom, Victoria.

PCR for the identification of fimbrial (F4, F5, F6, F41 and F18) and enterotoxin (StA, STb and LT) genes was carried out as previously described (Do et al., 2005). A negative control (all reagents except template DNA) and positive control (bacterial DNA from the reference strains) were included for each PCR. All PCR reactions were performed in duplicate.

SeroLOGY. A subset of 170 E. coli isolates was serogrouped at the National E. coli Reference Laboratory, Department of Primary Industries, Epsom, Victoria, Australia. Slide agglutination tests were performed using a set of O and OK rabbit antisera representing the most common porcine E. coli serogroups (O8, O8G7, O9, O20, O64, O101, O138:K81, O139, O139:K82, O141:K85ac, O141:K85ab, O147:K89, O149:K91: F4) as well as four F-antisera (F4, F5, F6 and F41). For the detection of fimbrial antigens, the colonies of tested strains were cultured overnight on 5 % SBA (for F4 and F6 testing) and Minca agar (for F5 and F41 testing) and subjected to Staphylococcus aureus coagglutination using rabbit antiserum specific for the different somatic and fimbrial antigens. An E. coli isolate was identified as an ETEC or putative ETEC strain if it possessed genes encoding specific colonizing fimbriae (F4, F5, F6 and F41) and/or enterotoxins (StA, STb and LT).

Statistical analysis. The statistical significance of differences in the incidence rates of diarrhoea and isolation of serogroups and pathotypes of ETEC were assessed between piggeries using the
RESULTS

Incidence of pre-weaning diarrhoea

Over the 6 month study, 4986 piglets born alive from a total of 568 litters farrowed on the five CPs were investigated to determine the incidence of diarrhoea (Table 1). Of the 568 litters farrowed, diarrhoea was observed in 406 litters, i.e. a mean litter incidence rate of 71.5% for the 6 month period of study. The rates were higher in piggeries A, B and E (71.5–79.2%) than in piggeries C and D (63.1–67.9%). However, the difference was not significant (P > 0.05). Of the 4986 piglets born alive, 3088 developed diarrhoea before the age of weaning (61.9%). The incidence rate of individual piglets with diarrhoea was significantly higher (P < 0.001) in piggeries A and B ( > 60%) compared to the other piggeries.

Prevalence of ETEC in piglets with diarrhoea

A total of 406 specimens (372 faecal and 34 intestinal content specimens) were submitted from piglets with diarrhoea at the five CPs and examined for the presence of ETEC. A total of 200 E. coli isolates satisfied the selection criteria of heavy, pure or nearly pure growth on SBA and MCA (49.2%), of which 126 were subsequently identified as ETEC by PCR, accounting for 31% of the total number of specimens. ETEC were isolated from a total of 43% of cases of diarrhoea in piglets 1–4 days of age and 23.9% of the remaining cases of diarrhoea up until the age of weaning.

A total of 62 E. coli isolates were obtained from specimens submitted to the NIVR diagnostic laboratory from VP sources during the same time period. Of these, 44 isolates (71%) were confirmed as carrying at least one porcine ETEC virulence gene.

Table 1. Results of a 6 month survey on pre-weaning piglet diarrhoea in five CPs (> 100 sows each) in north Vietnam

<table>
<thead>
<tr>
<th>Piggery</th>
<th>No. of litters born</th>
<th>No. of litters with diarrhoea (incidence rate)</th>
<th>No. of liveborne piglets</th>
<th>No. of piglets with diarrhoea (incidence rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>96</td>
<td>76 (79.2)</td>
<td>883</td>
<td>546 (61.8)*</td>
</tr>
<tr>
<td>B</td>
<td>249</td>
<td>178 (71.5)</td>
<td>2291</td>
<td>1510 (65.9)*</td>
</tr>
<tr>
<td>C</td>
<td>53</td>
<td>36 (67.9)</td>
<td>435</td>
<td>230 (52.9)</td>
</tr>
<tr>
<td>D</td>
<td>84</td>
<td>53 (63.1)</td>
<td>680</td>
<td>389 (57.2)</td>
</tr>
<tr>
<td>E</td>
<td>86</td>
<td>63 (73.3)</td>
<td>697</td>
<td>413 (59.3)</td>
</tr>
<tr>
<td>Total</td>
<td>568</td>
<td>406 (71.5)</td>
<td>4986</td>
<td>3088 (61.9)</td>
</tr>
</tbody>
</table>

*P < 0.001 (compared amongst piggeries A, B, C, D and E) (two-tailed Chi-square test).
(F4/STa/STb/LT, F4/STa/STb, F5/STa and F−/STb) were found amongst the ETEC isolated from VPs, and all these pathotypes were also detected in CP isolates. Except for the F−/STa/STb/LT pathotype, which was detected in a high proportion of CP isolates but not in any of the VP isolates, no significant difference (P > 0.05) in the prevalence of pathotypes was observed between CP and VP isolates.

Relationships between piglet age groups and ETEC serogroups and pathotypes

The distribution of ETEC isolates among different age groups of piglets in relation to serogroup, pathotype and the presence or absence of haemolysis is shown in Table 3. All isolates of the F4/STa/STb/LT pathotype showed haemolysis on SBA and belonged to serogroup O149:K91, which was the predominant ETEC serogroup detected in all three age groups of piglets. Interestingly, the second most prevalent was found to be F−/STa/STb/LT isolates that belonged to serogroup O8. These isolates also were haemolytic and distributed in all age groups of piglets. All remaining isolates belonged to the other serogroups and were non-haemolytic.

O8 : G7 isolates that belonged to pathotype F4/STa/STb were exclusively identified in the youngest age group of piglets (1–4 days of age). Serogroup O101 pathotype F4/STa/STb isolates were only identified in piglets 5–21 days of age, but not in the immediate neonatal period. The serogroup O64 pathotype F5/STa strains were only isolated from piglets up to 14 days of age. None of the F−/STb isolates belonged to any of the tested O serogroups. However, among the non-typable isolates, seven still harboured the F4 gene

Table 2. Fimbrial and enterotoxin genes detected in ETEC isolates of CP and VP origin

Data are presented as no. (%).

<table>
<thead>
<tr>
<th>Origin of isolate</th>
<th>Fimbrial genes</th>
<th>Enterotoxin genes</th>
<th>Subtotal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>STa</td>
<td>STb</td>
</tr>
<tr>
<td>CP (n = 126)</td>
<td>F4</td>
<td>25 (19·8)</td>
<td>42 (33·3)</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>21 (16·7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−</td>
<td>10 (7·9)</td>
<td>28 (22·3)</td>
</tr>
<tr>
<td>VP (n = 44)</td>
<td>F4</td>
<td>8 (18·2)</td>
<td>20 (45·5)</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>12 (27·3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−</td>
<td>4 (9·1)</td>
<td></td>
</tr>
</tbody>
</table>

*Negative for F4, F5, F6, F41 and F18.

Table 3. Frequencies of O serogroups associated with pathotypes and haemolysis patterns in ETEC isolates

Data are presented as no. of ETEC isolates from CPs (no. of ETEC isolates from VPs). ONT, O non-typable with the ten O and OK antisera used.

<table>
<thead>
<tr>
<th>Age of piglets (days)</th>
<th>Virulence genes</th>
<th>No. of haemolytic isolates</th>
<th>O serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>O149 : K91</td>
</tr>
<tr>
<td>1–4 (n = 80)</td>
<td>F4/STa/STb</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>F4/STa/STb/LT</td>
<td>30</td>
<td>18 (12)</td>
</tr>
<tr>
<td></td>
<td>F5/STa</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STa/STb/LT</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STb</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5–14 (n = 66)</td>
<td>F4/STa/STb</td>
<td></td>
<td>2 (8)</td>
</tr>
<tr>
<td></td>
<td>F4/STa/STb/LT</td>
<td>23</td>
<td>15 (8)</td>
</tr>
<tr>
<td></td>
<td>F5/STa</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STa/STb/LT</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STb</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>15–21 (n = 24)</td>
<td>F4/STa/STb</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>F4/STa/STb/LT</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STa/STb/LT</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F−/STb</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
and six had the F5 gene together with one or more enterotoxin genes. Serogroups O8 : G7 (pathotype F4/STa/STb) and O8 (pathotype F-/STa/STb/LT) were exclusively associated with isolates from CPs and were detected on all five farms.

DISCUSSION

The rapid expansion of the Vietnamese pig industry from smallholder farms with less than 20 sows to larger, more intensive piggeries, has resulted in the cross-breeding of imported pig breeds with local breeds. Under such conditions, it is possible that unique ETEC serogroups and pathotypes that were present in indigenous breeds of pig may have been selected and become widely distributed in larger piggeries. The current study was therefore undertaken to determine the significance of ETEC as a cause of pre-weaning diarrhoea at five selected large CPs in north Vietnam and to compare ETEC serogroups and pathotypes between CP and VP sources.

Identifying the major ETEC serogroups and pathotypes responsible for pre-weaning diarrhoea in north Vietnam was the first step in implementing a management and control plan for ND based on vaccination of sows using endogenous antibodies. It is possible that unique ETEC serogroups and pathotypes are associated with isolates from CPs and were detected on all five farms.

Although vaccination has been suggested to result in shifts in fimbrial antigens associated with ETEC in pigs (Garabal et al., 1997; Guinee & Jansen, 1979; Moon & Bunn, 1993; Noamani et al., 1999; Ojeniyy et al., 1994). Identification of ETEC that possess all three enterotoxins may indicate the emergence of a new and more virulent F4 ETEC pathotype (Fairbrother, 1999b).

The most noteworthy finding of this study was the relatively large number of ETEC isolates of CP origin (28 isolates, 22.3%) that belonged to serogroup O8, produced all three enterotoxins (STa, STb and LT) and did not possess any of the five recognized fimbriae (F-). This serogroup and pathotype was haemolytic, distributed in all age groups of pig in all five studied piggeries and was the second most prevalent behind serogroup O149 : K91 strains that were F4 positive. Interestingly, the F-/STa/STb/LT pathotype was not present in any isolates from CPs. The large number of F-/STa/STb/LT isolates could reflect the previous use of ETEC whole-cell bacterins containing F4 alone or in combination with the other fimbrial types associated with ND in the CPs. This would also explain the absence of the F-/STa/STb/LT pathotype in isolates from VP isolates raised by smallholding farmers, who do not regularly use vaccines for the control of ETEC infection, although it is possible that the F-/STa/STb/LT may have been detected in VP isolates if more isolates had been examined. It also is conceivable that the F- strains may have been present in the gastrointestinal tract of pigs imported into Vietnam. However, this unique pathotype has not been reported in Australia (Driesen et al., 1993), which exported live pigs to Vietnam, or in any other pig-producing country.

Although vaccination has been suggested to result in shifts in fimbrial antigens associated with ETEC in pigs (Garabal et al., 1997; Guinee & Jansen, 1979; Moon & Bunn, 1993;
Soderlind et al., 1982, 1988), these have been minor and short-lived, and despite extensive vaccination with F4ac vaccines in the USA and Europe this antigenic type remains dominant among ETEC implicated in diarrhoea in most countries (Moon, 1990; Westerman et al., 1988; Wittig & Fabricius, 1992; Moon & Bunn, 1993), as well as in the present study. The high proportion of O8 isolates expressing F−/STa/STb/LT could in part explain the apparent failure of previous prophylactic vaccination programmes in CPs in Vietnam. However, as ETEC were only found to be responsible for 31% of cases, this may not be as significant as the influence of other causes of pre-weaning diarrhoea in older age groups of piglets such as TGE, rotavirus and coccidiosis. Other factors, such as inappropriate sow, gilt and piglet management during farrowing and early lactation, may also contribute to the high incidence of diarrhoea in suckling pigs in Vietnam.

The O8 (F−/STa/STb/LT) and O149 (F4/STa/STb/LT) strains were both haemolytic and isolated from all age groups of piglets at each of the five CPs. Similar strains that lack recognized fimbiae belonging to O8 and additional serogroups, including O101, O139, O138, O141, O147, O149 and O157, have been noted in studies from other countries, including Japan (Negawa et al., 1987), Sweden (Soderlind et al., 1988) and Denmark (Ojeniyi et al., 1994). However, it is unusual to find the production of LT enterotoxin associated with strains other than F4- or F18-positive ETEC (Harel et al., 1991; Ojeniyi et al., 1994; Wilson & Francis, 1986), with a few notable exceptions (Brosø et al., 1988; Lund et al., 1982). This makes the O8 strains identified in the present study even more unusual in that they possess LT in association with STa and STb enterotoxins. This strongly suggests that unlike the non-typable isolates that were only shown to carry STb, the O8 isolates are very likely to be pathogenic and could possess an as yet undetermined novel colonization factor. In support of this hypothesis, the O8 (F−/STa/STb/LT) strains possessed a unique mannose-resistant haemagglutination property, were shown by electron microscopy to express fimbiae and were capable of attachment in vitro to isolated porcine brush borders. In addition, a representative isolate caused hypersecretory diarrhoea and was only found to be attached to the ileum of challenge-exposed, colostrum-deprived 1-day-old piglets (Do et al., 2006).

The presence of serogroup O7/O157 strains expressing a new adherence factor (AIDA-I) in combination with other ETEC virulence genes (AIDA/STb/EAST1 or AIDA/STb) has been confirmed in piglets with diarrhoea in Canada. This prompted the suggestion that AIDA-I plays a possible role in the pathogenesis of ETEC-associated diarrhoea in piglets (Ngeleka et al., 2003). The relatively high frequency of O8 (F−/STa/STb/LT) strains in CPs in north Vietnam together with their confirmed pathogenic potential in 1-day-old piglets provides an incentive for further studies to investigate their origin and the genetic basis of attachment. Furthermore, the inclusion of these unusual strains in ETEC vaccines together with the other common F4 and F5 pathotypes in Vietnam may confer better protection to piglets against ETEC infection during the pre-weaning period.

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