Characterization of pathogenicity determinants in *Escherichia coli* strains isolated from weaned piglets in Vietnam.

H. NGUYEN NGOC, C. TASCA, M. BOURY and A. MILON

SUMMARY

Two hundred and forty nine *Escherichia coli* strains isolated from weaned piglets in Vietnam (38 from mesenteric lymph nodes of piglets with clinical signs of edema disease (ED), 107 strains from faeces of piglets experiencing post-weaning diarrheas and 104 from faeces of healthy contemporaries) were tested by PCR for the presence of a set of pathogenicity determinants including genes encoding verotoxins (VT1, VT2, VT2e), enterotoxins (ST-I, ST-II and LT-I), adhesins (F4, F5, F6, F41 and F18) and the attaching/effacing strains marker intimin (Eae). Among the ED isolates, 13 strains (34.2 p.100), including twelve O139 and one O141 displayed VT2e and F18 positive genotype and were verocytotoxic *in vitro*, and 11 strains harboured an enterotoxigenic equipment that included ST-II and/or LT-I and sometimes F4 (6 strains). Typical verotoxicogenic ED (VT2e and F18 positive) strains were scarcely isolated from faeces of weaned piglets (5.7 p.100 from diarrheic and 1.9 p.100 from healthy ones) and all of these isolates harboured also ST-I, ST-II and LT-I genes, but none belonged to O138, O139 or O141 serogroups. Enterotoxigenic markers, with combinations of ST-I, ST-II and LT-I, were also recovered in 6.5 p.100 and 15.4 p.100 of the strains from diarrheic and healthy pigs respectively, without association to F4 or F18. In these strain collections, the adhesins F5, F6, F41, the intimin Eae and VT1 were never present. Our results suggest that ED in Vietnam is mainly due to O139 strains, but that verotoxicigenic/enterotoxigenic strains belonging to other serogroups are circulating in herds and may be a source of mobile genetic elements for typical adapted ED inducing strains.

KEY-WORDS: *Escherichia coli* - adhesins - verotoxins - enterotoxins - Vietnam - weaned piglets.

RÉSUMÉ

Caractérisation des marqueurs de pathogénicité de souches d’*Escherichia coli* isolées de porcelets sevrés au Vietnam. Par H. NGUYEN NGOC, C. TASCA, M. BOURY et A. MILON.

Deux cent quarante-neuf souches d’*E. coli* isolées de porcelets sevrés au Vietnam (38 de nœuds lymphatiques mérenténiques de porcelets présentant des signes cliniques de maladie de l’œdème (MO), 107 des fèces de porcelets présentant des diarrhées post-sevrage, et 104 des fèces de porcelets sains contemporains) ont fait l’objet d’une recherche par PCR des gènes codant pour les vérotoxines VT1, VT2 et VT2e, les entérotoxines ST-I, ST-II et LT-I, les adhésines F4, F5, F6, F18 et F41 et l’intimine (Eae). Parmi les isolats de MO, 13 souches (34.2 p.100) dont 12 de sérogroupes O139 et une O141 présentaient les marqueurs typiques VT2e et F18 et étaient verocytoxiques in vitro. Onze souches de cette collection présentaient des marqueurs de souches entérotoxigènes : ST-II et/ou LT-I et parfois F4 (6 souches). Des souches VT2e/F18 typiques de MO n’ont été isolées que chez 5,7 et 1,9 p.100 des porcelets diarrhéiques et sains respectivement et tous ces isolats présentaient en outre les gènes ST-I, ST-II et LT-I, mais aucun n’appartenaient aux sérogroupes O138, O139 ou O141. Des combinaisons de ST-I, ST-II et LT-I ont également été retrouvées respectivement chez 6,5 et 15,4 p.100 des isolats de porcelets diarrhéiques et sains, sans association à F4 ou à F18. Dans ces collections, F5, F6, F41, Eae et VT1 n’ont jamais été mis en évidence. Nos résultats suggèrent que la MO au Vietnam est principalement associée à des souches de sérogroupe O139, mais que des souches vero- et entérotoxigènes n’appartenant pas aux sérogroupes classiques O138/O139/O141 circulent dans les porcaries et pourraient être des sources d’éléments génétiques mobiles pour des souches typiques et adaptées, inducrices de MO.

MOTS-CLÉS : *Escherichia coli* - adhesines - verotoxines - enterotoxines - Vietnam - porcelets sevrés.

Introduction

Edema disease had been observed for the first time by Shanks in 1938 and up to now this disease is likely one of the important causes of the economic losses in pig husbandry throughout the world. The disease takes place most frequently during the post weaning period and is due to verotoxicigenic *E. coli* (VTEC) strains. Numerous studies have shown that the *E. coli* responsible for this disease belong to mainly to the O138, O139 and O141 serogroups [19]. These serogroups may be isolated from diarrheic or normal piglets too (3). *E. coli* strains isolated from edema disease cases are characterized by the production of a variant of verotoxin (VT) called VT2e, which is directly involved in edematous and neurological clinical signs, and by an adhesin, the fimbrae F18 (formerly F107), which permits colonization of the swine digestive tract (3, 26). However, another *E. coli* pathovar, the enterotoxigenic one (ETEC) may be isolated in wea-
ned piglets. Though ETEC are mainly involved in neonatal diarrheas, it may occasionally be found in postweanig diarrheas and/or enterotoxicosis. These ETEC strains are characterized by production of thermostable (ST) and/or thermolabile (LT) enterotoxins and several adhesins, such as F4 (K88), may be involved in their ability to colonize the digestive tract (45, 31, 35). In Vietnam, edema disease has been observed for the recent years. E. coli strains belonging to the serogroups O138, O139 and O141 have been isolated from the mesenteric lymph nodes of piglets clinically suspected of edema disease and originating from traditional as well as from industrial herds (33). But the pathogenicity determinants of these isolates were still unknown.

Our study aims at determining how these classical serogroups E. coli involved in edema disease distribute and which pathogenicity characters may be identified in isolates from different categories of Vietnamese piglets (i.e. suspected of edema disease, diarrheic and healthy weaned piglets).

Materials and methods

A) BACTERIAL STRAINS AND CULTURE

Two hundred and forty nine E. coli isolates were used for the study: 107 and 104 strains isolated from faeces of the diarrheic and healthy piglets respectively, and 38 strains isolated from the mesenteric lymph nodes of piglets suspected of edema disease. The E. coli strains were obtained from 4 geographically separated pig farms (i.e. without any commercial relationships) in south Vietnam between 1999 and 2001. The bacteria were isolated either from rectal swabs (diarrheic piglets and healthy piglets) or from pulp of the mesenteric lymph nodes (piglets suspected of edema disease, diarrheic and healthy weaned piglets). The strains were cutured on NA (Nutrient Agar, Difco) and directly cultured on EMB (Eosin Methyl Blue, Difco). The strains were cutured on NA (Nutrient Agar, Difco) and directly cultured on EMB (Eosin Methyl Blue, Difco).

B) SEROTYPING

The strains were cutured on NA (Nutrient Agar, Difco) and serotyped by slide agglutination using 3 antisera: O138: K81, O139: K82 and O141: K85 (LDA22, Ploufragan, France).

C) PATHOGENICITY FACTORS DETERMINATION.

Polymerase chain reactions (PCR) were used to detect the presence of genes encoding the following virulence factors: VT1, VT2, VT2e, ST-I, ST-II, LT-I, Eae, F4, F5, F6, F41 and F18. The primers used are presented in table 1.

Genomic DNA was prepared from E. coli bacteria grown in Luria Bertani (LB) broth (Difco) cutured at 37°C for 24h with shaking. Bacterial culture (100 µl) was pelleted at 12,000 g for 3 min, resuspended in 25 µl NaOH 0.5N for 20-30 min for bacterial lysis, then 25 µl Tris HCl 1M pH 7.4 and 450 µl H2O were added.

For the detection of genes encoding the virulence factors, single or multiplex PCR were used. The primers were synthesized by Isoprim company (Toulouse). The PCR was made with Taq DNApolymerase (Gibco BRL) in a total reaction mix of 50 µl which contained 5 µl of genomic bacterial DNA, 0.2 mM of each dNTP, 0.5 µl of each primer, 2 U of Taq DNA polymerase, 2 mM MgCl2, 5 µl reaction buffer 10X and H2O.

One drop of mineral oil was added over the mixture of PCR. Amplifications were carried out with the following programme: hot start at 94°C for 5 min followed by 25 cycles of 45s at 94°C, 45s at temperature which was 3 to 4 degrees lower than that of theoretically calculated Tm of the primers, and 1 min at 72°C. The final extension step was performed at 72°C for 10 min.

The analysis of amplified products was performed by electrophoresis in 0.7 p.100 agarose gels (Sigma) containing ethidium bromide (5 µg/ml) in TBE buffer, run at 80V. A 1kb or 100bp molecular size ladder (depending on the size of the amlicon) was included in each analysis.

C) VEROCYTOTOXICITY ASSAYS

The bacterial strains were inoculated into 5 ml LB and incubated for 24h at 37°C with shaking. Supernatants from cultures centrifuged at 10,000 g for 3 min were filter-sterilized (0.22 µm) and ten fold serially diluted in cells culture medium in 96 wells flat-bottomed microtiter plates. Dilutions of culture filtrate were added to Vero cells cultures. Vero cells (african green monkey kidney, ATCC CCL 81) were grown at 37°C in Eagle’s minimal Essential medium (EMEM) with Earle’s salts (Gibco/BRL) supplemented with 10 p.100 heat inactivated fetal bovin serum (Gibco/BRL), 80 µg /ml gentamycin and 5 mM glutamin. Vero cell monolayers were established by growth at 37°C, in a 5 p.100 CO2 atmosphere. Confluent monolayers were harvested and diluted in cell culture medium prepared as above to 3.106 cells/ml and dispensed in 200 µl aliquots into flat-bottomed 96 wells microtiter plates. The plates were incubated at 37°C, under 5 p.100 CO2 atmosphere for 24h, then used for the test. The ten-fold dilutions (50 µl) of each bacterial supernatant filtrate were added to each well of the plates and incubation was continued. Vero cells were observed daily for five days by light microscopy for cytotoxicity. After five days of incubation, the cells were fixed by formalin 3.7 p.100 for 1h, then stained with methylene blue 1 p.100 for 1h at laboratory temperature. For calculating the median cytotoxic dose CD50, the stained Vero cells were carefully washed and the stain that remained in live cells was removed with HCl and quantified by spectrophotometer at 630 nm. The median cytotoxic dose CD50 was calculated with the software CrickGraph.

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Results

A) E. COLI SEROGROUPS.

Serotyping of the strains by slide agglutination (Table II) showed that 16 out of 38 (42.1% p.100) E. coli strains from mesenteric lymph nodes of piglets suspected of edema disease belonged to serogroup O139, 12 out of 38 (31.6% p.100) belonged to O141, and 2 out of 38 (5.3% p.100) belonged to O138 (table II). On the contrary, only 8 (7.7% p.100) and 1 (0.9% p.100) out of 107 strains from healthy piglets belonged respectively to O139 and O141 serogroups. In weaned piglets with diarrhea, 2 strains out of 104 reacted with the O138 antiserum (table II).

B) ANALYSIS FOR PATHOGENICITY DETERMINANTS BY PCR

All strains were screened by single or multiplex PCR for the presence of genes encoding verotoxins (VT1, VT2 and the specific B sub-unit of VT2e), enterotoxins (ST-I, ST-II and LT-I) and fimbrial adhesins (F4, F5, F6, F18, F41). The gene eae, that encodes intimin, a marker of the Locus of enteroxyte effacement which is encountered in Attaching/Effacing E. coli (including enteropathogenic and enterohemorrhagic strains) has been searched too. The results are presented in table II, and samples of agarose gel electrophoresis are given in figure 1.

No strains scored positive for VT1, intimin, F5, F6 or F41. The absence of eae in that set of strains confirms that pig VTEC are not part of EPEC/EHEC pathovars, or more generally that they do not belong to the Attaching/Effacing E. coli group.

Mesenteric lymph nodes isolates, gathered from piglets dead or euthanized with clinical signs of ED, diplayed two types of genetic equipment : (i) typical ED VTEC set of genes (VT2/VT2e positive reactions indicating that they possess the genes for the VT2e variant, and F18 adhesin) and (ii) typical ETEC equipment, that includes at least one enterotoxin and the adhesin F4, or occasionally F18. The ED VTEC recruited mainly in the O139 serogroup, with 13 out of 16 isolates, one of them possessing ST-II, LT-I and F4 too. Only one O141 isolate out of 12 bring VT2e and F18, while 5 of these had an ETEC equipment. None of the O138 (2 strains) or non typeable strains (8 isolates) possessed VT2e. However, 5 out of 8 NT strains had an ETEC genotype. It should be stressed that F18 (fedA gene) was detected in the absence of VT2e in 3 isolates (1 O141 and 2 NT). All strains possessing F4 adhesin were found LT-I and ST-II-positive.

In the set of E. coli strains isolated from the faeces of normal or diarrheic weaned piglets (table II), only 31 out of 211 isolates possessed at least one gene encoding for a toxin (VTEC or ETEC). The ratios were not different between isolates from diarrheas (13/107) or not (18/104). All these strains were non typeable with the 3 O:K antisera (O138,
O139, O141) and, on the contrary, none of the O138/O139/O141 isolates (11/211) harboured any pathogenicity determinant. Eight of NT strains were VT2e-producing and F18-positive, with six strains isolated from diarrheic and 2 from healthy piglets. Interestingly, all of them harboured also ST-I, ST-II and LT-I genes. The majority (29/31) of strains possessed ST-II gene. Half of strains were positive for both ST-I and ST-II.

**C) VEROTOXICITY ASSAYS**.

The verocytotoxicity of the VT2e positive strains was confirmed by cell culture assays using lysates or culture supernatants of clinical isolates from mesenteric lymph nodes (figure 2). The verocytotoxicity of the culture supernatants was found moderate, ranging from $10^3$ to $10^4$ Vero CD$_{50}$ ml$^{-1}$. The lysates of the isolates were 100- to 1000-fold more toxic than the supernatants. All isolates that scored positive for VT2e by PCR were found cytotoxic for Vero cells.

For the isolates from faeces of the healthy or diarrheic pigs, only culture supernatants were tested in cytotoxicity assays. Verotoxicity of these strains was 10-fold or 100-fold more than that of supernatants of the clinical isolates from mesenteric lymph nodes (data not shown).

**Discussion**

The VTEC strains responsible for edema disease possess two characteristic virulence factors: the verotoxin VT2e and the *fimbriae* F18 [1, 17, 27]. However, strains carrying genes encoding the factors of colonization F4, F5, F6, F41 or enterotoxins: ST, LT which are characteristic for ETEC may be recovered from weaned piglets and responsible for post-weaning diarrheas or enterotoxicosis. VTEC are also responsible

<table>
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<th>Origin of isolates</th>
<th>Serogroup (N° of strains)</th>
<th>VT2</th>
<th>VT2e</th>
<th>ST-I</th>
<th>ST-II</th>
<th>LT-I</th>
<th>F18</th>
<th>F4</th>
<th>N° of strains (p.100)</th>
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a : Strains isolated from Mesenteric Lymph Nodes of piglets with clinical signs of ED.

b : Not typeable, i.e non O138/O139/O141 strains.

**TABLE II.** — PCR results obtained from strains isolated from mesenteric lymph nodes of piglets with edema disease, and from fecal samples of diarrheic or healthy piglets in Vietnam. No strains scored positive for VT1, eae, F5 (K99), F6 (987P) and F41.
of major food-born diseases in humans: strains of enterohemorrhagic (EHEC) pathotype, carrying VT1 and/or VT2 and eae are involved in infections resulting in haemorrhagic colitis or haemolytic and uremic syndromes (HUS) [2]. However, these strains are very scarcely recovered from pigs as compared to bovine [18]. The E. coli strains which produce VT2e usually belong to serogroups O138, O139, O141 and cause edema disease in pigs [13, 19]. These strains are not invasive, but may recovered from the mesenteric lymph nodes, where they can translocate during agony of the animals or shortly after death [33, 42, 33]. In this work, 38 strains were isolated from 38 mesenteric lymph nodes of 38 piglets that died with clinical of edema disease. Most of them (30/38) belonged to serogroup O138, O139, O141, and cause edema disease in pigs [13, 19]. These strains are

**Figure 1.** — PCR results for genotyping of ED isolates.

A: Multiplex reactions for detection of VT2 (amplicon size 807 bp), Eae (570 bp), VT1 (388 bp) and VT2e (261 bp). Lane 1: Reference strain EHEC EDL933 (eae, VT1, VT2), 2 to 9: ED isolates (7 of them being positive for VT2/VT2e in lanes 2-8), 10: K12 DH5α (negative control). Lane 11: 1kb ladder (size markers).

B: Single PCR reactions for search of F4 (lanes 1 and 2, amplicon size: 601 bp), 987P (4 and 5: 333 bp), F41 (7 to 9: 380 bp) and F5 (lanes 11 to 14: 314 bp). Lanes 1, 4, 7 and 11: K12 DH5α DNA (negative control), lane 2: T25 ED isolate (F4 positive), lanes 5 and 14: P4271 control strain (987P pos, F5 neg), lanes 8, 9, 12, 13: B41 control strain (F41 and F5 positive).

C: Multiplex reaction for ST-II (amplicon size: 172 bp) and LT-I (696 bp). Only DNA from ED isolates were run in this gel, with positive results in lanes 4, 8, 9 (ST+, LT+) and 5 (LT+ only).
lower than that found by DA SILVA et al., [6], or WASTESON et al., [45] who showed that the prevalence of VT2e in ED isolates to be 68.8 p.100 to 100 p.100. It is possible that non verotoxigenic strains from the normal flora as well as VTEC may invade, at the same time, the mucosa and mesenteric lymph nodes after death. It must be stressed that these authors have worked on faecal isolates which may be excreted at high level during the acute phase of disease and therefore isolated more easily in the samples.

NGUYEN N.H. et al., 2000 [33] showed that the majority of the strains isolated from the mesenteric lymph nodes of the piglets suspected of ED in Vietnam were O139 and O141, which occurred with prevalences of 12.1 p. 100 and 42.9 p. 100 respectively. In this study, these serogroups were also well represented, with 42.1 p. 100 and 31.6 p. 100, respectively. In Belgium, POHL P. et al., [40] found the majority of the strains to be O141 and O138 or O139 were not found. The majority of the E. coli VT2e positive strains in our study were O139 (92.3 p. 100) which was in good agreement with the results of DA SILVA A.S. et al., [6] or WITTIG et al., [47].

The association of the genes encoding for VT2e et F18 is very frequent in strains responsible for edema disease [19, 36]. Our results of PCR showed this association in 13 out of 16 strains (81.3 p. 100) isolated from ED, 12 belonging to the O139 serogroup. The results are in good agreement with WITTIG W. et al., [47]. All these strains were found verocytotoxic in vitro, as also described by MARQUES et al. [27].

The genes encoding enterotoxins (ST-I, ST-II and LT-I) and factors of colonization characteristic for ETEC (Enterotoxigenic E. coli) may also be found in the VT2e positive E. coli [4, 45, 14, 15]. Our study shows that only 1 out of /3 VT2e-positive strains was also positive for ST-II and LT-I. None of the strains of this collection possessed the gene encoding for ST-I. And only 4 out of 38 (10.5 p. 100) isolates from piglets suspected of ED were positive for F4 (1 strain belonged to O139 serogroup and 3 others belong to O141). OSEK [36] and WITTIG W. et al. [47] didn’t found those genes in any E. coli strains belonging to O138, O139 and O141.

FAIRBROTHER et al. [8] and FRYDENDAHL K. [10] found the gene encoding for Intimin (eae) in 9.3 p. 100 and 1.4 p. 100 of the E. coli strains isolated from the piglets with diarrhoea or edema. In our collections, no strains scored positive for VT1 or eae, (thus belonging to the enterohaemorrhagic -EHEC- pathotype) and all strains that scored positive for VT2 were also positive for the VT2e specific B subunit. Though the nucleotide sequences of VT2 and VT2e are very homologous [17, 39, 43, 46], the genes encoding for their B subunit can be easily distinguish by PCR with specific primers for VT2e [17], as shown in fig 1. Our results are in agreement with that of many authors [2, 5, 26, 44] and confirm that pig is not a major source of EHEC for human beings.

**FIGURE 2.** — Cytotoxicity of supernatants and sonic lysates from cultures of VT2e positive strains from mesenteric lymph node. Data presented are the mean or results from 13 isolates. The dilutions 1/1000 (10^{-3}) of supernatants and 1/100,000 (10^{-5}) of lysates contain approximately one cytotoxic dose 50 p.100 (CD50).
Among 211 *E. coli* strains from healthy and diarrheic weaned piglets, no strain scored positive for F4, F5, F41 or F6. This is in good agreement with the work of Osek [35, 36, 37] who showed that these genes are scarcely present in *E. coli* strains isolated from weaned piglets. These adhesins are more characteristic for *E. coli* isolated from diarrheas in neonate suckling piglets than for strains responsible for post-weaning colibacillosis [11, 19, 47].

VTEC strains may also be isolated from healthy and diarrheic weaning pigs, but at lower frequencies than from pigs suffering from the ED [34, 35, 36, 47, 30]. Our study in Vietnam confirms that point. In our hands, as far as the major serogroups are concerned, 1.87 p. 100 of the *E. coli* isolates from the diarrheic piglets were O138 and we did not find any strain O139 nor O141. Among the strains isolated from healthy piglets, 8.7 p. 100 of the isolates were O139 or O141. In Ojeniyi's study [34], the serogroups O138, O139 and O141 were also found with low frequencies: (10.0 p. 100, 2.9 p. 100 and 7.1 p. 100 respectively) and similar results (10.9 p. 100) were obtained by GANNON et al., [14]. However, in our study, none of the O138/O139/O141 strains found in this collection were positive for VT2e and/or F18, or any of the pathogenicity markers that were tested. On the contrary, 5.6 p.100 (6 strains out of 107) and 1.9 p. 100 (2 out of 104) of the untyped faecal strains, isolated respectively from the diarrheic and healthy animals, were positive for VT2e, F18 genes and also for genes encoding the enterotoxins ST-I, ST-II and LT-I. It is known that VTEC from pig cannot be uniformly assigned to the O138/O139/O141 serogroups. For instance, WOODWARD et al. have showed *E. coli* isolates or swine origin to belong to serogroups O9, O26, O65 and to have VT2e but not F18 [48]. Enterotoxigenic markers, with combinations of ST-I, ST-II and LT-I, were also recovered in 6.5 p.100 and 15.4 p.100 of the strains from diarrheic and healthy pigs respectively, without association to F4 or F18. It is known that most adhesin and toxin genes are carried by mobile genetic elements such as plasmids or phages. It is thus possible that these untyped strains may serve as reservoirs of pathogenicity genes that may be transmitted to classical, adapted clones that may induce pathologies in weaned animals.

In Argentina, 6.3 p. 100 of *E. coli* isolates from healthy piglets were found VT2e positive [38], GANNON [14] got 14.0 p.100 of VTEC from healthy and diarrheic piglets in Canada. OSEK, [35] in Poland and WOODWARD et al., [48] in UK showed the prevalences of VTEC from diarrheic piglets with to be 9.1 p.100 and 8.5 p.100 respectively. The percentage of VTEC were higher in the studies of NAGY et al., [32] and WITTIG et al., [47] with 25.0 p.100 and 48.7 p.100 respectively. The differences of in the prevalences of VTEC isolated from the piglets between the countries may be explained by the local husbandry management system. Highly integrated systems that are used in developed countries lead to the easy dissemination of pathogenic clones of bacteria along with the trade of animals. The Vietnamese situation lies more in local production systems, with few animal exchanges. This situation may explain the low dissemination of classical VTEC serogroups.

To conclude, the analysis of *E. coli* isolates from weaned pigs shows that ED is mainly associated with classical strains belonging to O139 (and sometimes O141) serogroup, but some untyped VTEC/ETEC strains can also be found circulating among diarrheic and healthy weaned piglets. These results indicate that serotyping is not sufficient to characterize ED-inducing or more generally colibacillosis-inducing isolates in Vietnam and that PCR is a reliable and quick method to complete diagnosis. Our results also confirm that VTEC from pig do not carry typical EHEC genes such as *stx1, stx2* or *eae* and thus cannot be involved in food-borne diseases leading to haemorrhagic colitis and HUS in humans.

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**Reference**


