1. Introduction

Intestinal adhesion and subsequent colonization are believed to be the key initial steps by which pathogenic *E. coli* establishes infection. Some enteropathogenic *E. coli* (EPEC) strains adhere to or invade Hep-2 and HeLa cell monolayers [18]. Some strains of enterohaemorrhagic (EHEC) pathotype have been reported to adhere to tissue culture; however, EHEC adherence to cells in vitro is frequently poor [6]. Postweaning diarrhoea and edema disease of swine are diseases due to colonization by enterotoxigenic *E. coli* (ETEC) or verotoxigenic *E. coli* (VTEC), respectively. Porcine ETEC produce enterotoxins, while VTEC strains produce a variant of the Shiga-like toxin (verotoxin VT2e), which is directly involved in the pathogenesis of the disease, including edemas and neurologic signs. Some strains can produce both enterotoxins and verotoxin (ETEC/VTEC). Some of the ETEC or VTEC strains that lack classical adhesins like K88 (F4), K99 (F5), F41 or 987P (F6) fimbriae have been shown to express F18 (formerly F107) adhesin that helps colonizing orogastrically infected weaned pigs [7, 14]. The physiopathology of colonization was similar to that of VTEC O139:K12:H1 strains associated with edema disease [1].

Pigs are commonly affected by VTEC immediately after weaning. The strains O139 colonized the small intestines of experimentally inoculated pigs and adhered in vitro to porcine enterocyte brush border fragments [1]. The F18ac...
strains adhered in vivo to ligated intestinal loops in weaned pigs, while the F18ab strains did not adhere or adhered weakly and similarly in vitro with the isolated intestinal brush borders from weaned pigs [3]. Some strains of E. coli serogroup O139 could adhere to Hep-2 cell independently to the presence of F18-encoding genes [16].

The adhesion tests using swine intestinal villi or brush borders to demonstrate the fimbrial expression of E. coli strains, including the fimbrae F18, is expensive and time-consuming. The preparation of villi or enterocytes is difficult, and moreover the results of the test depend not only on the preparation of the substrate but also on the sensitivity of the piglets, which varies with age and genotype [17, 4, 5, 11, 21, 2, 22, 19, 8, 10].

HeLa cells, a cell line from human epithelial origin are frequently used as cellular supports for the phenotypic adhesion tests of E. coli in vitro. For instance, several authors have shown that EPEC could bind to HeLa cells [13, 18, 20] but that ETEC or EIEC (Enteroinvasive E. coli) could not [20].

The aims of our work were to find whether the enterotoxemia VT2e+ and F18+ isolates from piglets with edema disease would bind in vitro to cultured cell lines. For this purpose, we tested the pattern of adhesion of a set of VTEC isolates to HeLa cells, and to LLCPK-1 (a cell line from pig kidney origin), as compared to adhesion to swine intestinal villi and in relation to the presence of genes encoding the fimbrae F18, the characteristic colonization factor of the VTEC responsible for edema disease in piglets.

### 2. Materials and methods

#### A) BACTERIAL STRAINS AND CULTURE

Twenty two E. coli strains isolated from piglets suffering from edema disease or post-weaning diarrheas in Vietnam were used. All strains were serogrouped and characterized by PCR for the genes encoding VTs, ST-I, ST-II, LT-I, F4, F5, F6, F18 and F41. Interesting properties are listed in Table I for each strain. Briefly, as far as adhesins are concerned, all strains were negative for F5, F6 and F41. Fifteen belonged to serogroup O139, 12 being positive for VT2e and F18 and 3 negative for both markers: 4 strains of serogroup O141, 1 being positive for VT2e and F18, 1 being positive for only F18 and 2 being positive for F4 were also used. Finally, 3 untypable strains (i.e. non O138, O139 nor O141), 1 of them being positive for F18 and VT2e, 1 for F18 only and 1 being positive for F4 were tested. All strains possessing the genes encoding VT2e were cytotoxic for Vero cells in vitro. The strain DH5α (K12) and the rabbit EPEC strain E22 (O103:K-H2) were used as negative and positive controls in cell-lines adhesion tests. Pig isolates and the strain DH5α were grown in Trypsinase soy broth (TSB, Difco) medium and E. coli E22 was grown in Penassay medium (antibiotic medium 3, Difco) [13] overnight at 37°C with shaking.

#### B) ADHESION TESTS ON HeLa AND LLCPK-1 CELLS

HeLa 229 (ATCC ref. CCL2) and LLCPK-1 (ATCC ref. CL-101) cells were grown at 37°C under 5% CO2. For

<table>
<thead>
<tr>
<th>Strains</th>
<th>Serogroups, genotypes</th>
<th>Adhesion substrate</th>
<th>LLCPK-1</th>
<th>HeLa</th>
<th>Swine intestinal villi</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>“sensitive”</td>
<td>“resistant”</td>
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<tr>
<td>H28</td>
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<td>-</td>
<td>-</td>
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<tr>
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<tr>
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<td>NT</td>
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<tr>
<td>B22</td>
<td>Untypable, F4+</td>
<td>-</td>
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</tbody>
</table>

* : not tested. **untypable : not belonging to serogroups O138, O139, O141

**Table I.** — Results of in vitro adhesion tests of E. coli strains isolated from piglets in Vietnam. Genotypes were tested by PCR. All strains were negative for F5, F6 and F41.
adhesion tests, suspensions of 5.10^4 cells in 500 µl of Dulbecco’s Minimum Essential Medium (GIBCO) supplemented with 10 p. 100 fetal calf serum, 10mM glutamin and 80 µg/ml gentamycine were seeded in eight-chamber Lab-Tek slides. Cells were cultured for 20 hours. Before tests, cells were washed 2 times with phosphate buffered saline (PBS), then 470 µl of interaction medium (MEM containing 2 p.100 of fetal calf serum and 1 p.100 D-mannose) were added to the chambers. Twenty µl of overnight bacterial culture was then added to each chamber and the slides were incubated for 30 minutes at 37°C under 5 p. 100 CO₂.

The chambers were then washed 5 times with PBS. The cells were fixed with methanol for 20 minutes and stained with Giemsa for one hour. The stain was washed with demineralized water, dried, and examined under a light microscope. In routine, the results of adhesion tests was subjectively evaluated by the density of bacteria binding to the cells for 25 cells : ± : less the 5 bacteria binding per cell ; + : 5 - 20 bacteria binding per cell ; ++ : more than 20 bacteria binding per cell.

C) PREPARATION OF SWINE INTESTINAL VILLI

Ileal villi samples were prepared from 18 to 24 day-old piglets with two types of sensitivity to edema disease. The piglets considered as «resistant» originated from herds where just a few clinical cases of edema disease appeared under a high infection pressure whereas piglets considered as «sensitive» originated from herds where there were a lot of cases of edema disease. The ileal villi were prepared by the method described by GIRARDEAU J.P., 1980 [reported in 13]. Briefly, piglets were euthanized under anaesthesia, and 50 cm of ileum were quickly sampled and immersed into Krebs buffer (NaCl 0.12M, KCl 0.014M, NaHCO₃ 0.025M, NaH₂PO₄ 0.01M, pH 7.2) at 0°C. The villi were harvested by scratching out ileal surface into the buffer. The villi suspensions were washed several times with 1 p. 100 formalin buffered solution and finally kept at -80°C in HANKS-DMSO-Glycerol conservation medium (HANKS salt solution 40 p. 100, fetal calf serum 10 p. 100, dimethylsulfoxide 30 p. 100, glycerol 20 p. 100).

D) ADHESION TEST ON SWINE INTESTINAL VILLI

One ml of ileal villi suspensions were thawed at laboratory’s temperature and then diluted in 15 ml of HBSS (Hank’s Balance Salt Solution, Gibco). The villi suspensions were washed three times in HBSS before used. One hundred µl of villi suspensions were seeded in each well of 96 wells round-bottomed microtiter plates with enlarged top cones to avoid damaging the villi, then 25 µl of each bacterial culture were added to the wells and plates were incubated at 37°C for 30 min with gentle shaking. Each strain was tested several times with «sensitive» and «resistant» villi. After interaction, villi were observed under phase contrast microscopy. Adherent strains are easy to identify by their adherence to the highly refringent brush border of enterocytes. The result of adhesion’s test was subjectively estimated by the presence and density of the bacteria binding to the villi, from «heavy» (+) when all brush borders appeared colonized to «slight» (±), when adherent bacteria could be easily but scarcely distinguished.

2. Results

A) ADHESION TESTS ON THE HeLa AND LLCPK-1 CELL LINES

Results are summarized in table I. Fourteen out of 16 (87.5 p. 100) F18-positive strains could adhere onto HeLa cells in presence of D-mannose, whatever their serogroup, i.e. O139 (12/13), O141 (1/1), or untypable (1/2). The density of bacteria ranged from good (++ : 8/14), to slight (± : 2/14) and the pattern of adhesion was always diffuse and looked alike that of the EPEC E22 (fig. 1). On the contrary, none of the strains could adhere to LLCPK-1 cells. Three F4-positive strains (B1, B20 and B22) did not show any adhesion on HeLa or LLCPK-1 cells. None of F18/F4-negative strains (strains H43, T20 and T12) could adhere onto HeLa cells in presence of 1 p. 100 of mannose.

B) ADHESION’S TEST ON SWINE INTESTINAL VILLI

All strains that were tested in vitro on HeLa and LLCPK cells, except the strains H43, T20, T12 and DS51, were also tested for their adhesive capacity on porcine ileal villi. The results (table I) showed that 87.5 p. 100 (14/16) of the F18-positive strains could adhere in vitro to the villi prepared from edema-disease «sensitive» piglets but not to the «resistant» ones (figure 2), except one untypable strain (B37), which showed a slight adhesion. The adhesion intensity varied depending on the strains. A comparison of the adhesion test’s results involving the different cellular supports (table I) showed that there was a close correlation between the adhesive capacity of the F18-positive strains on HeLa cells and on «sensitive» intestinal villi.

3. Discussion

The utilization of three adhesion substrates : HeLa cells, LLCPK-1 cells and porcine intestinal villi prepared from 18 - 24 day-old piglets allowed to characterize the adhesive capacity and adhesion pattern of the F18-positive E. coli strains isolated from piglets suspected of edema disease in Vietnam. These strains could adhere well, in vitro, to HeLa cells and to the porcine intestinal villi from edema-disease «sensitive» piglets (figure 1 and 2), but could not adhere to the LLCPK-1 pig kidney cell line. The intensity of adhesions to HeLa cells and villi were well correlated (table I).

The mannose-resistant diffuse or localized adhesions to HeLa cells have been reported in strains of EPEC (enteropathogenic E. coli) [18, 13] or EHEC (enterohemorrhagic E. coli) pathotypes [6], but that of ETEC (enterotoxigenic E. coli), EIEC (enteroinvasive E. coli) or UPEC (uropathogenic E. coli) strains were not [21]. Some E. coli porcine strains belonging to serogroup O139 have been reported to display mannose-resistant adherence to Hep-2 cells without relation-
ship with the presence of the F18 genes [17]. But, up to now, the adhesion of the VTEC F18-positive porcine strains to HeLa cells has not been reported. In our experiment, it is evident that the VTEC F18-positive strains responsible for edema disease in piglets could adhere to the HeLa cells. However, this capacity varies with the strains, and probably with the culture conditions that may modulate the expression of the fimbriae F18 by the bacteria. Several authors have frequently reported a discordance between the fimbrial expression in vitro and in vivo [3, 24, 9, 10]. In our study, 14 out of 16 F18-positive strains that displayed adhesion on HeLa cells were negative for all the genes encoding the other classical colonization factors that characterize *E. coli* strains originating from pigs, such as: F4, F5, F6 and F41. Moreover, in our experiments, the F18-negative and F4-positive strains couldn’t adhere to HeLa cells. So, it is possible that the *fimbriae* F18 was the colonization factor responsible for the adhesion of these strains onto HeLa cells. The culture conditions that have been used for the strains may have permitted a good expression of F18 and the ability to adhere without needing to create micro-aerobical conditions as described by WITTING et al. [25]. Nevertheless, our study cannot exclude that other adhesins, yet to be identified, may have a role in this adhesion. On that point, further work is needed, and other tests such as neutralization of the adhesion phenotype by F18-specific antibodies have to be undertaken.

BERSTCHINGER H.U. et al., [2] VOGELI P. et al., [22, 23] have shown that the predisposition of animal to intestinal colonization by F18ab-positive *E. coli* is genetically controlled by dominant alleles of a putative locus encoding the F18 receptor on porcine enterocytes (ECF18R). This locus has been located on the band q11 of swine chromosome 6, close to two alpha [1, 2] fucosyltransferase genes, which polymorphism may be used to type the animals [12]. It means that there are 2 types of the susceptibilities of enterocytes to the adhesion of the *E. coli* responsible for edema disease in piglets, and that the susceptibility to adhesion may give insight to sensitivity of piglets to edema disease. We tried to test this hypothesis by using, in adhesion tests, intestinal villi derived from two genetically different types of piglets, originating from herds with low («resistant») or high («sensitive») disease prevalence under the same infectious pressure. Though piglets from which the villi were gathered have not been strictly genotyped, our results show that the adhesion of the F-18 positive *E. coli* strains happened only on «sensitive» porcine intestinal villi.

There are two antigenic types of the *fimbriae* F18 in the *E. coli* isolated from post-weaning piglets: F18ab which characterizes *E. coli* responsible for edema disease and which is prevalent in the O139 serogroup, and F18ac which is more often found in *E. coli* responsible for post-weaning diarr-

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**Figure 1.** — Diffuse adhesion patterns on HeLa cells. A : strain DH5α (K12) (negative control). B : EPEC strain E22 (positive control). C : T22 isolate (O139, VT2+, F18+) showing a ++ pattern. D : H29 isolate (O139, VT2+, F18+) showing a + pattern.
rhoeas and in O141 strains [25]. NAGY B. et al. [15] have shown that the biologic properties of these fimbrae are different: F18ab expresses worse, in vitro and in vivo, than F18ac, and the F18ac-positive E. coli adhere on porcine intestinal villi better than the F18ab-positive E. coli. F18 antigenic variants have not been typed in our strains, however, 12/13 (92.3%) O139 F18-positive strains could adhere to porcine intestinal villi. This discrepancy may be due to in vitro culture conditions that may modulate expression of the F18 fimbrae or to the animal susceptibility to F18. The same reasons may explain that F4 positive strains did not adhere to intestinal villi in our hands, though F4 receptor on enterocytes should be expressed in animals within the age range of the piglets we used to prepare the villi [8, 19].

To conclude, our results show that the VTEC F18-positive strains isolated from the piglets suspected of edema disease in Vietnam could adhere to HeLa cells in presence of D-mannose and with a diffuse pattern. The F18 adhesin is very likely involved in this adhesion phenotype as (i) the phenotype correlates quite well with the presence of the fedA gene, as detected by PCR, (ii) no other known adhesin genes have been detected in the strains showing the adhesion phenotype (including F4, F5, F6 and F41), (iii) the adhesion phenotype on HeLa cells correlates with adhesion to intestinal villi prepared from piglets supposed to be sensitive to edema disease, but not to villi from «resistant» piglets. However neutralization tests with antibodies against F18 must be done to ascertain the role of F18 fimbrae in this adhesion phenotype. As a whole, HeLa cells may be proposed as an easy-to-handle substrate to check the presence and/or expression of F18 on VTEC strains isolated from edema diseased piglets, as compared to the intestinal villi or enterocytes preparations, which are expensive and time-consuming.

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References


12. — MEIJERINK E., FRIES R., VOGELI P., MASABANDA J., WIGGER G., STRCKER C., NEUENSCWANDER S., BERTSCHINGER H.U. and STRANZINGER G. : Two alpha (1,2) fucosyltransferase genes on porcine chromosome 6q11 are closely linked to the blood group inhibitor (S) and F18R receptor : ECF fimbriae. Infect. Immun., 1999, 67, 520-526.


