Detection and identification of pathotypes of verocytotoxigenic *Escherichia coli* isolated from weaned piglets using gene probes for seven *E. coli* toxins

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1. SUMMARY

Seventy verocytotoxigenic (VTEC) and sixty-three non VTEC haemolytic *Escherichia coli* isolated from recently weaned piglets were examined by the colony hybridization assay using gene probes for three verocytotoxins: Edema disease principle (EDP) and Shiga-like toxins I and II (SLTI and SLTII). The results with the EDP and SLTII probes were identical. All VTEC hybridized with these two probes, while non VTEC did not. All 133 *E. coli* were negative for the SLTI probe. Hybridization of the plasmid content of 14 VTEC did not show any evidence for plasmid localization of the genes coding for the EDP. The 70 VTEC were also assayed with gene probes for heat-stable (STaP, STb) and heat-labile (LT, LTIIa) enterotoxins. Only the STb probe was hybridized by 36 of them. Most STb-positive isolates

belonged to serotype O141 : K85 biotypes 9 and 13 PC.

2. INTRODUCTION

Edema disease (ED) occurs in recently weaned piglets and is associated with *Escherichia coli* producing a heat-labile toxin that is (i) responsible for the lesions (ii) neurotoxic for mice and (iii) cytotoxic for Vero cell cultures [1–4]. Most of these verocytotoxigenic porcine *E. coli* (VTEC) are haemolytic and belong to the O138 : K81, O139 : K82, O141 : K85 serotypes [4–6]. Some of these verocytotoxigenic *E. coli* (VTEC) can also produce one or more of the classical enterotoxins (Ent VTEC isolates) and for this reason are thought to be also involved in post-weaning diarrhea [4,6–8].

The verocytotoxin produced by ED-associated *E. coli*, or *Edema Disease Principle* (EDP), is related to the family of Shiga and Shiga-like toxins (ShT, SLTI or VTI, SLTII or VTII) of *Shigella dysenteriae* type 1 and of *E. coli* [9]. ShT and SLTI are biologically identical, antigenically simi-

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lar and genetically highly related. SLTII is also biologically identical to ShT and to SLTI, but differs antigenically and genetically from them. EDP is biologically distinct from ShT and from the SLTs by its lack of toxicity for HeLa cell cultures [10,11] but is antigenically and genetically related to SLTII [8,11–13]. For these reasons, EDP has also been named SLTII variant (SLTIIv) [12]. The genes coding for ShT are located on the chromosome while the synthesis of the SLTs is phage-mediated in many isolates that have been studied. Presence of the genes coding for EDP on the chromosome has been observed in two isolates [12,13].

The diagnosis of ED is based on clinical signs and lesions and is usually confirmed by the demonstration of large numbers of haemolytic *E. coli* in piglet intestines. However, not all the haemolytic *E. coli* are VTEC nor do all VTEC produce haemolysin [4,6,14]. It is not easy to demonstrate the production of a verocytotoxin when a large number of *E. coli* must be studied. A rapid and sensitive diagnostic test is needed for the detection of porcine VTEC associated with ED.

Recent studies have raised the possibility of using the colony hybridization assay with an SLTII (or VTII) probe [8,15] as a laboratory assay for the detection of porcine *E. coli* associated with ED. Moreover, Gyles [13] has cloned the gene for the EDP and derived a gene probe that is specific for VTEC associated with ED, but also hybridizes with SLTII-positive *E. coli*.

The goals of the work reported here were (1) to compare the results of the probing of porcine VTEC and non VTEC with the EDP, SLTI and SLTII probes; (2) to complete the pathotypes of the VTEC using gene probes for four other *E. coli* toxins (STaP, STb, LT, LTIIa); and (3) to look for a possible plasmid localization of the genes coding for the EDP by plasmid hybridization.

3. MATERIALS AND METHODS

3.1. Bacterial strains

One hundred and thirty-three haemolytic *E. coli* isolated from weaned piglets were included in the study. Seventy of them produce a cytotoxin

Table 1

Deviation of the gene probes for seven *Escherichia coli* toxins

Probe name [reference]	Recombinant plasmid	Endonuclease *	Size of the probe (base pairs)
EDP [13]	pCG6	*Acc*I + *Pst*I	900
SLTI [15]	pJN37-19	*Bam*HI	1142
SLTII [15]	pNN111-19	*Pst*I	842
STaP [21]	pRIT10130	*Hin*fI	157
STb [17]	pRAS1	*Hin*fI	460
LT [17]	pEWD299	*Hinc*I	800
LTIIa [22]	pCP2725	*Hin*dII + *Pst*I	800

* Restriction endonuclease used to derive the gene probe fragment from the recombinant plasmid.

active on Vero cells (VTEC isolates) and 63 do not (non VTEC isolates) [14]. Their serotypes and biotypes have been reported elsewhere [14]. Forty-three of the VTEC belong to the O141 : K85 serotype; of them, 22 to the 9PC biotype, 13 to the 13PC biotype and eight to the 14PC biotype. The remaining 27 VTEC could not be assigned to the usual serotypes associated with ED (NT isolates).

3.2. Gene probe derivation

Gene probes for the seven *E. coli* toxins were derived from the recombinant plasmids listed in Table 1 after purification by the alkaline lysis method and by ultracentrifugation in cesium chloride [16]. Plasmids were digested by appropriate restriction endonucleases (Table 1), and the generated fragments were separated by agarose or acrylamide gel electrophoresis. The gene probe fragments (Table 1) were removed from the gel by electroelution [16]. They were subsequently radio-labelled with [^{32}P]-deoxycytidine triphosphate using a multiprimer labelling kit (Amersham Belgium).

3.3. Filter hybridization assay

The bacteria to be assayed, along with the *E. coli* K12 and wild type control strains, were inoculated onto nutrient agar following the grid shown in Fig. 1, transferred by contact onto Whatman 541 paper filters and treated by alkaline lysis [17]. Filters were placed colonies side up onto Whatman 3MM paper filters saturated with the following solutions: (i) 10% SDS (sodium dodecylsul-
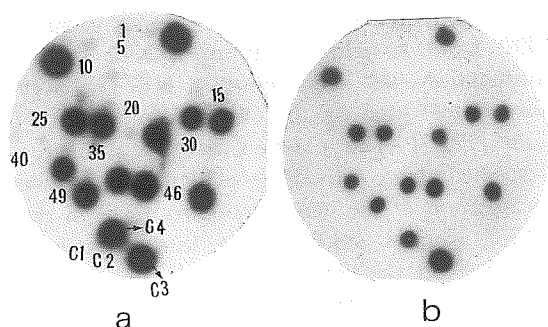
Fig. 1. Comparison of results obtained with EDP (a) and SLTII (b) probes on some of the VTEC and non VTEC isolates. Black spots represent colonies hybridizing with the gene probes. 1 to 50: wild type *E. coli* isolates; C1: SLTI probe positive control; C2: negative control for the different probes; C3: SLTII probe positive control; C4: EDP probe positive control.

fate) for 3 min, (ii) 0.5 M NaOH/1.5 M NaCl for 15 min, (iii) 0.5 M Tris HCl (pH 7.5)/1.5 M NaCl twice for 5 min each time. Filters were subsequently hybridized overnight with the different gene probes at 65°C in a solution comprising 3 × SSC (0.45 M NaCl/0.045 M Na citrate), 10 × Denhardt solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone 360, 0.2% bovine serum albumin), 1% salmon sperm DNA, and 0.1% SDS (18). Next day, filters were washed three times for 30 min each time at 65°C in a solution comprising 3 × SSC and 0.1% SDS, dried and autoradiographed.

3.4. Plasmid hybridization assay

The plasmid DNA present in 14 VTEC was extracted according to Kado and Liu [19]. The different plasmids were separated by electrophoresis overnight at 4°C in a 0.5% agarose gel. The DNA within the gels was denatured and the gels were dried [18]. The gels were subsequently hybridized with the appropriate gene probes in the same conditions as on the filters.

4. RESULTS

4.1. Hybridization of the K12 and control strains

All seven probes hybridized with their corresponding *E. coli* K12 and control strains. Hybridi-

zation of the SLTII probe by the EDP-producing *E. coli* wild type control strain and by the *E. coli* K12 strain containing the cloned gene for EDP was observed. Similarly, the SLTII-producing *E. coli* wild type control strain and the *E. coli* K12 strain containing the cloned gene for the SLTII were also positive using the EDP probe.

4.2. Hybridization of the VTEC and non VTEC isolates with the EDP, SLTI and SLTII probes

The results on the wild type isolates with the EDP and SLTII probes were identical (Fig. 1). All 70 VTEC isolates were positive and all 63 non VTEC isolates were negative with these two probes. The VTEC isolate previously found to be SLTII probe-negative [14] was positive with the EDP and SLTII probes when tested in this study. All 133 *E. coli* were negative with the SLTI probe.

4.3. Hybridization of the VTEC isolates with gene probes for four other toxins (STaP, STb, LT, LTIIa)

Three of these gene probes (STaP, LT, LTIIa) were not hybridized by any of the 70 VTEC isolates. Thirty-six of them (52%) hybridized with the STb probe (EDP + SLTII + STb + pathotype). The other thirty-four VTEC isolates (48%) showed the EDP + SLTII + pathotype.

Thirty (70%) of the O141:K85 VTEC isolates hybridized with the STb probe but only 6 (22%) of the NT VTEC isolates. All but one (95%) of the O141:K85 VTEC isolates with the 9PC (95%) and 62% of those with the 13PC were STb + but only one with the 14PC (12%). The NT VTEC isolates were too few to make any comparison between the different biotypes.

4.4. Hybridization of the plasmids extracted from 14 VTEC with the EDP and SLTII probes

The results of these hybridizations showed that none of the plasmids extracted had homology with the EDP and SLTII probes in the conditions used.

5. DISCUSSION

ED is usually associated with haemolytic VTEC [4,6,14], but not all haemolytic *E. coli* isolated from recently weaned piglets are VTEC [6,14]. The

cytotoxic assay on Vero cell cultures must be performed to confirm a diagnosis of ED. The production of EDP by *E. coli* can be distinguished from the production of SLTI and/or SLTII in three ways: (i) toxicity titers for Vero cells of EDP-producing cultures are usually much lower than the toxicity titers of SLTs-producing cultures [6,8], due either to a difference in the amounts of toxin produced, or a difference in the cytotoxicity of the molecules; (ii) EDP is not active on HeLa cell cultures in contrast to SLTs [10,11]; (iii) cytotoxicity of EDP is not neutralized by antibody to SLTI, but it is by antibody to SLTII [8,11], although there is some controversy in the literature [7].

As these phenotypic assays on Vero cells are time-consuming and difficult to carry out on a large number of *E. coli* isolates the DNA colony hybridization genotypic assay using gene probes for EDP, SLTI and SLTII was performed with VTEC and non VTEC porcine isolates. The results of this work show that (i) EDP and SLTII probes cross-hybridized (ii) both EDP and SLTII probes hybridized to all VTEC but to none of the non VTEC isolates (iii) all *E. coli* isolates were negative using the gene probe for SLTI which does not seem to be common among porcine *E. coli* [6,7,20]. The genes encoding EDP show extensive homology with those encoding SLTII, but not with those encoding SLTI [12,13]. This homology between the genes coding for EDP and for SLTII results in cross-hybridization of probes that are derived from them, and in indistinguishable hybridization results on wild type porcine *E. coli*.

This study would thus demonstrate the need for more specific gene probes for EDP and SLTII in order to avoid any possible confusion between EDP-producing and SLTII-producing porcine VTEC isolates. However, the production of SLTII by porcine *E. coli* has been reported for only one isolate so far [6]. Thus, the colony hybridization assay using the EDP probe represents an alternative and reliable procedure for the detection of porcine VTEC isolates and for the confirmation of a diagnosis of ED, and is also less time-consuming than the cytotoxic assay.

The presence of genes encoding EDP on plasmids or on phages has not yet been demonstrated [4,11]; although the transfer of genes coding for EDP from a field isolate to a K12 strain by conjugation has been reported [4]. These genes have been found on the chromosome in two isolates [12,13]. The plasmid hybridization results described above provide no evidence for a plasmid localization of the genes encoding EDP in the 14 VTEC isolates studied, and thus suggest their presence on the chromosome, without excluding the possibility of their location on a prophage.

Some VTEC isolates associated with ED also produce one or more classical enterotoxins (Ent VTEC isolates) [4,6–8]. The Ent VTEC isolates belonging to the O141:K85 serotype usually produce STb alone ([6], see also RESULTS). Moreover, these STb + O141:K85 VTEC isolates are much more common in certain biotypes than in others. Ent VTEC isolates rarely belong to serotypes other than the three classical ones associated with ED ([6], this work).

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