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The prevalence and characterization of verotoxin-producing *Escherichia coli* isolated from cattle and pigs in an abattoir in Hong Kong

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SUMMARY

The aim of the study was to define the prevalence of verotoxin-producing *Escherichia coli* (VTEC) in cattle and pigs in a Hong Kong abattoir. Faecal and carcass samples collected from 986 cattle and 487 pigs from an abattoir were tested for verotoxin (VT) by PCR and cytotoxicity assays. VTEC was isolated from 41·5 and 1·8% of cattle faecal and carcass samples and from 2·1 and 0·2% of porcine faecal and carcass samples, respectively. Amongst 409 VTEC isolates from cattle, 9 were serotype O157:H7 and $eaeA^+$. The most prevalent *vt* genotype among bovine VTEC was $vt1^+vt2^+$ (73·8%) and in porcine VTEC was $vt2e^+$ (30%). None of the porcine VTEC isolates and 9·3% of the bovine VTEC isolates was $eaeA^+$. The non-O157 serogroup VTEC isolates carrying *eaeA* and *EHEC-hlyA* belonged to serogroups O172, O15, O84, O91, O110 and O121. The local dietary preference for pork or chicken (rather than beef), the low VTEC carriage in pigs, the rarity of additional virulence factors (*eaeA*) in VTEC isolated from cattle may explain the apparently low incidence of human diarrhoeal disease associated with VTEC in Hong Kong hitherto. However, the presence of non-O157 VTEC strains carrying the *eaeA* virulence marker in cattle highlights the fact that sole reliance on sorbitol-MacConkey agar for screening human VTEC isolates may underestimate the human disease burden. The changing dietary habits of the population in Hong Kong reinforce the need for continued vigilance.

INTRODUCTION

Verocytotoxin (VT) is a potent cytotoxin that inhibits eukaryotic protein synthesis and is a well-established virulence factor for *Escherichia coli* (VTEC) associated with human disease [1]. VTEC are geographically widespread and have been found to be associated with human disease ranging from bloody diarrhoea to the haemolytic-uraemic syndrome (HUS) [1]. The pathogenicity of VTEC is associated with additional virulence factors, the best known being the attaching-and-effacing (*eae*) A gene that encodes intimin, a 94 kDa outer-membrane protein [2]. Intimin enables VTEC to cause attaching-and-effacing lesions in the microvilli of the host intestine. Another virulence factor, the enterohaemorrhagic *E. coli* haemolysin A (*EHEC-hlyA*) gene, is encoded on a 60 MDa plasmid and is associated with EHEC strains [2]. Although Sandhu and colleagues reported that *EHEC-hlyA* was present in almost all *eaeA*-positive VTEC isolates obtained from animals [3], its role in the pathogenicity of VTEC is poorly understood.

VTEC is a naturally occurring organism in the gut flora of cattle [4] and has been isolated from the gut of other animals such as sheep, goats and pigs [1].

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Outbreaks are associated with consumption of contaminated undercooked ground beef and vegetables or unpasteurized milk [2]. Serotype O157: H7, the best-known serotype of VTEC associated with human disease, was responsible for the foodborne outbreak that occurred in Japan in 1996 in which a total of 9578 cases were reported [5]. DNA patterns of the isolates from infected cases suggested a heterogeneous source of contamination.

The epidemiology of VTEC in man and animals has been studied in Western countries and Japan [6–8] but data from South East Asia and China are sparse. In this study, faecal and carcass samples were collected from cattle and pigs in an abattoir in Hong Kong, in an attempt to study the frequency of VTEC carriage in healthy livestock in the province. Local abattoirs process over 2000000 pigs and 50000 cattle annually [9]. Since the local community consumes more pork than beef, the carriage of VTEC in pigs was of particular interest. Other virulence factors associated with VTEC strains isolated were also examined.

MATERIALS AND METHODS

Collection of faecal and carcass samples

Rectal and carcass swabs were collected immediately after the slaughter and evisceration of animals in a Government abattoir in Hong Kong. The animals sampled were 986 adult cattle and 487 adult pigs transported to Hong Kong immediately prior to slaughter from various provinces in mainland China. The samples were collected monthly between August 1996 and December 1998.

Culture for VTEC

Rectal swabs were inoculated onto sorbitol–MacConkey (SMAC) agar (Oxoid Ltd, Basingstoke, UK) and incubated at 37 °C overnight. Swabs from carcasses were inoculated into VTEC enrichment broth (2 g tryptone, 1·12 g bile salt, 5 g lactose, 4 g K_2HPO_4, 1·5 g KH_2PO_4, 5 g NaCl and 0·02 g novobiocin per litre) and incubated overnight with gentle agitation at 37 °C. A loopful of each broth culture was streaked onto a SMAC plate. One ml was centrifuged at 13000 r.p.m. in a microcentrifuge for 5 min and the bacterial cell pellet was used for PCR. Red or pink-coloured bacterial colonies on SMAC plates were regarded as sorbitol fermenting and colourless colonies as sorbitol non-fermenting.

Detection of *vt* genes by PCR and DNA hybridization

The bacterial pellet from each carcass broth culture and a colony sweep from the SMAC plate of each rectal swab was suspended in 0·5 ml sterile distilled water and heated at 80 °C for 15 min. The supernatant was used for PCR analysis using primers MK1 and 2 (Table 1) as described previously [10]. The 25 μl PCR mixture consisted of 3 μl supernatant, 50 mM KCl, 10 mM Tris–HCl (pH 8·3), 1·5 mM $MgCl_2$, 240 μM dNTP, 400 μg/ml BSA, 20 ρmole of each primer and 1 U Ampli*Taq* Gold DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT, USA). The PCR mixture was heated to 94 °C for 12 min and then subjected to 30 cycles of amplification (1 min at 94 °C, 3 min at 43 °C and 1 min at 72 °C respectively), followed by a final extension period of 7 min at 72 °C. Amplified products were visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel. PCR-amplified DNA was transferred from the agarose gel to Hybond-N nylon membranes (Amersham, Arlington Heights, IL, USA) by Southern blotting [11]. The membranes were hybridized at 45 °C overnight, with probes 428-I, 428-II and vt2e (Table 1) for detection of *vt1*, *vt2* and *vt2e* (GenBank accession number M29153 [12]) respectively. These probes were labelled using the DIG DNA 3′ end-labeling and detection kit (Boerhinger–Mannheim, Mannheim, Germany). The overnight hybridization was followed by two stringency washes of the membrane at 50EC in 0·2 × SSC (30 mM NaCl, 3 mM trisodium citrate, pH 7·0) with 0·1% SDS for 15 min. Labelled DNA was visualized by anti-digoxigenin enzyme-linked immunosorbent assay.

Detection of VT production by vero cell assay

Samples positive for *vt* by PCR were confirmed by a vero-cell cytotoxicity assay [13]. For each specimen, 10 colonies were picked from the SMAC plate and cultured in non-selective nutrient broth. One ml of each broth culture was centrifuged at 13000 r.p.m. for 15 min; 50 μl of supernatant was diluted 1 in 5 in Eagle's minimal essential medium and added to vero cell monolayers grown in 96-well microtitre plates. The plates were incubated at 37 °C overnight in 5% CO_2 and cells were examined for cytopathic effect (CPE) using an inverted microscope. Supernatants producing CPE of $\geqslant 50\%$ of the monolayer were considered VT producers.

Table 1. *Primers and probes used for detection of virulence genes of VTEC isolates*

	Target gene	Sequence (5′–3′)	Location (bp)	Reference
Primer				
MK1	*vt*	TTTACGATAGACTTCTCGAC	311–330	10
MK2	*vt*	CACATATAAATTATTTCGCTC	515–535	
hlyAF	*EHEC-hlyA*	GCATCATCAAGCGTACGTTCC	70–90	14
hlyAR	*EHEC-hlyA*	AATGAGCCAAGCTGGTTAAGCT	582–603	
C1	*eaeA*	TCGTCACAGTTGCAGGCCTGGT	803–824	15
C2	*eaeA*	CGAAGTCTTATCCGCCGTAAAGT	1890–1912	
Probe				
428-I	*vt1*	GATAGTGGCTCAGGGGATAA	428–441	10
428-II	*vt2*	AACCACACCCACGGCAGTTA	428–447	10
vt2e	*vt2e*	AATCATACACCACCAGGAAG	431–450	This study

Detection of *eaeA* and *EHEC-hlyA* genes in VTEC isolates

VTEC isolates confirmed by vero cell assay were studied for their *eaeA* and *EHEC-hlyA* genes by multiplex PCR. The primers were based on the central conserved region and the 5′ region of the respective genes [14, 15]. The PCR reaction mix was identical to that for *vt* gene detection. Amplification conditions were as follows: 94 °C for 12 min followed by 30 cycles of amplification for 1 min each at 94 °C, 55 °C and 72 °C. Primers and probes for detection and identification of virulence genes in VTEC are listed in Table 1. *eaeA*-positive strains were further confirmed by fluorescence actin stain (FAS), as described previously [13].

Serotyping of isolates

All VTEC isolates identified by vero cell assay were confirmed by traditional biochemical tests. All sorbitol non-fermenting strains were serotyped using the *E. coli* O157:H7 Latex Test kit (Remel, Lenexa, KS, USA). Non-O157 VTEC isolates harbouring *eaeA* and *EHEC-hlyA* genes were serotyped at the Microbiological Diagnostic Unit, Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia.

Pulsed-field gel electrophoresis of O157:H7 isolates

The method of Cameron and colleagues [16] was used with minor modifications in the preparation of DNA plugs. A small portion of the plug (2×7 mm) was digested with 16 units of restriction enzyme *Xba*I (Amersham Pharmacia Biotech, Uppsala, Sweden) at 37 °C overnight. Electrophoresis was performed in 1% agarose in $0{\cdot}5 \times$ TBE (12 °C buffer temperature) at 6 V/cm in a CHEF MAPPER system (Bio–Rad, Hercules, California, USA) with pulse times increasing from 5–50 s over a period of 20 h.

RESULTS

Prevalence and characteristics of VTEC in abattoir animals

VTEC were isolated from 409 (41·5%) faecal and 18 (1·8%) carcass samples from 986 cattle, and from 10 (2·1%) faecal and 1 (0·2%) carcass samples from 487 pigs (Table 2). Only four (0·41%) cattle yielded VTEC from both faeces and carcasses. PCR revealed a higher VTEC carriage in both species, particularly in carcasses (Table 2). The carriage rate in cattle appeared to be lower between May to August (data not shown) and highest in February and November.

Nine isolates of O157:H7 were isolated from cattle, one from a carcass and the remainder from faeces (Table 3). The O157:H7 isolate from the cattle carcass did not produce VT. All nine O157:H7 VTEC strains were sorbitol non-fermenting while 81% (324/401) of the non-O157 VTEC fermented sorbitol. In addition, one third of the non-O157 VTEC carrying principal virulence factors also fermented sorbitol. VTEC strains with *eaeA* were positive in the FAS test. Serotypes of all 30 non-O157 VTEC isolates carrying the principal virulence factors (*vt*, *eaeA* and *EHEC-hlyA* genes) isolated from cattle were determined and they were O172:H-, O15:H-, O91:H14, O84:H-, O110:H16, O121:H19, O rough:H-, O rough:H31 and O non-typeable:H- (Table 3).

Genotypic profile of the VTEC isolates

The genotypes of the VTEC isolates from cattle are summarized in Table 3. The most frequently isolated

Table 2. *Carriage rate of VTEC in cattle and pigs detected by PCR and vero cell assay*

	Cattle		Pigs	
	Faeces (No. = 986)*	Carcass (No. = 986)	Faeces (No. = 487)	Carcass (No. = 487)
PCR	62·5%	11·4%	5·1%	2·3%
Vero cell assay†	41·5%	1·8%	2·1%	0·2%

* No., number of samples.
† The percent values for vero cell assay are also the total numbers of VTEC isolates.

Table 3. *Genotypic profile of VTEC strains isolated from cattle*

Serotypes	Number of strains	*vt-1*	*vt-2*	*eaeA*	*EHEC-hlyA*	Verotoxicity
O157:H7	4	+	+	+	+	+
	4	−	+	+	+	+
O172:H-	10	−	+	+	+	+
O121:H19	1	+	+	+	+	+
O111:H16	1	+	+	+	+	+
O91:H14	2	+	+	+	+	+
O84:H-	1	+	+	+	+	+
O15:H-	2	−	+	+	+	+
O15:H-	2	+	+	+	+	+
O rough:H-	4	+	+	+	+	+
O rough:H31	1	+	+	+	+	+
O non-typable:H-	6	+	+	+	+	+
Other non-O157:H7	112	+	+	−	+	+
	25	−	+	−	+	+
	168	+	+	−	−	+
	64	−	+	−	−	+
	1	+	−	−	+	+
	1	−	−	−	+	+

Table 4. *Genotypic profiles of VTEC isolated from rectal swabs from pigs*

Number of isolates	*vt1*	*vt2*	*vt2e*	*eaeA*	*EHEC-hlyA*
2	+	−	−	−	+
1	−	+	−	−	+
1	−	+	+	−	−
1	−	+	−	−	−
1	+	+	−	−	+
1	+	+	−	−	−
3	−	−	+	−	−

bovine *vt* genotype was $vt1^{+}vt2^{+}$ (73·8%). Only one strain (0·24%) carried $vt1^{+}$ alone. One verotoxic isolate did not hybridize with the *vt1*-, *vt2*- or *vt2e*-specific probes. This isolate was also negative in PCR for *vt2c* (results not shown). The virulence markers $eaeA^{+}$ and $EHEC\text{-}hlyA^{+}$ were present in 9·3% and 43·3% respectively, of the bovine VTEC strains. Thirty non-O157 and eight O157:H7 strains harboured *vt*, *eaeA* and *EHEC-hlyA* genes. One O157:H7 isolate was non-verotoxic but carried both *eaeA* and *EHEC-hlyA* genes.

The genotypic profiles of VTEC isolated from pigs are summarized in Table 4. None of the porcine VTEC strains carried the *eaeA* gene and three strains carried *vt2e* gene alone. The most prevalent genotype was $vt2e^{+}eaeA^{-}$ $EHEC\text{-}hlyA^{-}$, three isolates collected during the same abattoir visit belonged to this genotype. The next most common genotype was $vt1^{+}eaeA^{-}$ $EHEC\text{-}hlyA^{+}$. Two isolates fell into this category and they were isolated during different abattoir visits (Table 4).

Pulsed-field gel electrophoresis of O157:H7 isolates

Seven PFGE patterns were identified from the nine O157:H7 isolates and were designated A–G (Fig. 1). Isolates recovered from different abattoir visits had

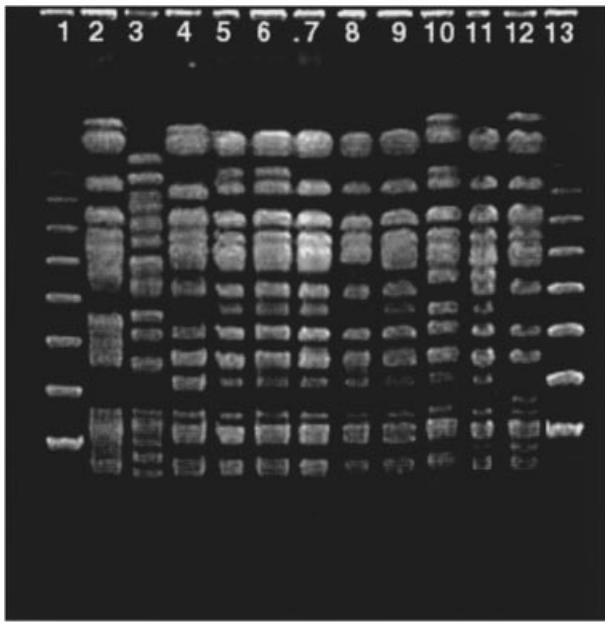

Fig. 1. Pulsed-field gel electrophoresis of O157:H7 isolates. Lanes 1 and 13, phage lambda DNA ladder size marker (Bio–Rad). Lane 2, O157:H7 (WHO strain). Lane 3, *E. coli* ATCC25922 (non-O157:H7 control). Lane 4, pattern A. Lanes 5 and 6, pattern B. Lanes 7 and 9, pattern C. Lane 8, pattern D. Lane 10, pattern E. Lane 11, pattern F. Lane 12, pattern G.

Table 5. *PFGE patterns and* vt *genotypes of O157:H7 strains isolated from cattle at various abattoir visits*

Date of abattoir visit	Number of samples	PFGE pattern	*vt* genotype
August 1996	1	A	No *vt* gene detected
April 1998	2	B	$vt1^{+}vt2^{+}$
November 1997	2	C	$vt2^{+}$
	1	D	$vt1^{+}vt2^{+}$
August 1998	1	E	$vt1^{+}vt2^{+}$
January 1997	1	F	$vt2^{+}$
June 1998	1	G	$vt2^{+}$

different patterns (Table 5), two isolates from a visit in April 1998 belonged to pattern B. Three isolates from a visit in November 1997 showed two PFGE patterns, C and D. The latter isolate had a different *vt* genotype.

DISCUSSION

In the present study, 41·5% of cattle harboured VTEC as determined by vero cell assay. The large discrepancy between PCR and the vero cytotoxicity assay may be partly accounted by the novobiocin-containing enrichment medium used for carcass samples, as the medium may be inhibitory to some VTEC strains [17]. In the evisceration process, the carcass is sprayed with hot water. This may kill contaminant bacteria and may explain the discrepancy between PCR (which can detect non-viable VTEC) and culture in the carcass samples. For the purposes of this study, only those specimens that were vero-cytotoxic were considered further.

The high carriage rate of VTEC in cattle also indicated that they were reservoirs for transmission of the organism. In other geographic areas, VTEC carriage rates in healthy cattle have been reported to be 34% in France [18], 24·3% in Japan [7], 53% in Sri Lanka [19] and 9·5% in Canada [8]. Our highest carriage rate occurred during the coolest months, which contrasts with the finding in France where the highest VTEC prevalence was in summer [18].

O157:H7 is the best known VTEC serotype associated with human diseases [2] and its presence in healthy animals and meat products has been reported by others [18, 20]. Here, the O157:H7 serotype was detected in 0·9% of the faecal samples from cattle, a rate comparable to that reported by Pradel and colleagues [18]. Eight O157:H7 isolates possessed the principal virulence factors (*vt*, *eaeA* and *EHEC-hlyA*). One of the isolates was vt^{-} but $eaeA^{+}$ and $EHEC\text{-}hlyA^{+}$, loss of *vt* gene probably occurred during subcultivation [21]. Nevertheless, $vt^{-}eaeA^{+}$ O157:H7 and O26:NM have been isolated from healthy and diarrhoeal animals respectively [4, 22] and the pathogenicity of such strains remains to be defined. The PFGE pattern of isolates collected during one abattoir visit (presumably from the same farm) was identical but there were diverse patterns of isolates from different abattoir visits (Fig. 1).

The predominant *vt* genotype has been shown to vary in different geographical locations [18, 19, 23, 24] but the most prevalent *vt* genotype encountered in our cattle was $vt1^{+}vt2^{+}$. In this study, one verotoxic strain did not belong to *vt1*, *vt2*, *vt2e* or *vt2c* and this isolate requires further study. The predominance of *vt2* (either alone or together with *vt1*) in our VTEC is noteworthy as VT2-producing VTEC were highly associated with HUS [25]. In our previous study on 1003 faecal samples from diarrhoeal patients in Hong Kong, of five VTEC isolated, two O157:H7 and one O rough:H45 harboured *vt2* [26]. In this study, the majority of bovine non-O157 VTEC isolates were negative for *eaeA*. Such $eaeA^{-}$ VTEC have been reported to cause HUS in humans [27, 28]. Human isolates in Hong Kong have included O rough:H45 and O157:NM $eaeA^{-}$ strains [26], indicating that $eaeA^{-}$ VTEC may harbour virulence factors outside

the locus of enterocyte effacement. Such potential virulence factors include the *astA* and *katP*, which encode for a heat-stable enterotoxin and a catalase-peroxidase respectively. These strains have been found in animals and asymptomatic human carriers [29].

Previous studies showed that the *EHEC-hlyA* genotype was more frequently isolated from haemorrhagic colitis (HC) and HUS patients, indicating that this gene might play a role in the disease process [2]. In 66 animal VTEC isolates, the *EHEC-hlyA* and *vt* genes were closely associated, serving as an useful epidemiological marker for rapid selection of VTEC [30]. However, only 43·3% of VTEC from our cattle carried the *EHEC-hlyA* gene.

Pigs were included in this study as pork is a major source of meat consumed by the local population. We found that both the VTEC carriage and carcass contamination rates in healthy pigs were low. Our finding agrees with that of other investigators [23, 24], suggesting that pigs are not an important reservoir of VTEC. The O157:H7 serotype was not isolated from pigs and the predominant genotype of the porcine VTEC was *vt2e*$^{+}$*eaeA*$^{-}$ *EHEC-hlyA*$^{-}$. The role of VTEC in human diarrhoea in Hong Kong is lower than that in other countries [6, 31]. One reason may be the absence of *eaeA* in porcine and the majority of the bovine VTEC. Another may be the local preference for eating well-cooked meats and pork rather than beef.

Non-O157 VTEC carrying the virulence factors *eaeA* and *EHEC-hlyA* were serotyped (Table 3). One-third of these strains belonged to serotype O172, which has been isolated from one patient with HC in America [32] but was infrequently encountered in both humans and animals elsewhere [33, 34]. The first reported O172 strains harboured *vt2* as did the O172 isolates described here. O15 is a common serotype in rabbit diarrhoea *E. coli* [35]. However, O15 strains can also be encountered in EHEC and are associated with cattle infection [36]. Serotype O91 is known to cause HUS in humans [27] and this serotype has been reported in healthy and diseased animals [37]. The O84, O110 and O121 serotypes have been isolated in diarrhoeal calves and humans [37–39]. This group of VTEC isolates may be potential intestinal pathogens causing a human public health hazard in Hong Kong and they may go unrecognized in human clinical specimens if reliance is placed solely on sorbitol–MacConkey agar for screening VTEC isolates. This, together with the changing dietary habits of the population reinforces the need for continued vigilance for outbreaks of VTEC in the humans.

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