Dual Infection with Enterotoxigenic *Escherichia coli* and Porcine Reproductive and Respiratory Syndrome Virus Observed in Weaning Pigs that Died Suddenly

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ABSTRACT. Diarrhea, sudden death after short duration of diarrhea and sudden death without apparent signs were observed in a herd of breeder pigs. Five pigs that died suddenly with diarrhea (SDD pigs) and 6 pigs that died suddenly without signs (SD pigs) were examined. The average age of the pigs was about 28 days. Twelve pigs of age 10 to 14 days old showing diarrhea (D pigs) were also examined. Eleven of them recovered. Large numbers of *Escherichia coli* were detected in all organs of every SDD and SD pig and in feces of D pigs. All of the isolates were identified as enterotoxigenic *E. coli* (ETEC) by the polymerase chain reaction (PCR). Porcine reproductive and respiratory syndrome (PRRS) virus cDNA was also detected from the lung of every SD and SDD pig by the RT-PCR. High and low titers of antibodies to PRRS virus were found in 10-day-old and 1-month-old pigs, respectively. In an experiment, 3 ETEC were isolated from 9 healthy weaning pigs during the quiescent stage in the herd. These data showed that growth of the ETEC was not active in healthy weaning pigs; however, following infection with PRRS virus ETEC infection became systemic and caused peracute death in the weaning pigs. It suggested also that infection with PRRS virus in 10-day-old pigs were protected by the colostral antibodies, and fatal infection by ETEC did not occur as a result. — KEY WORDS: enterotoxigenic *E. coli*, PRRS virus, sudden death, swine (weaning).

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Diarrhea in piglets is known to occur frequently in the new born and weanling [6, 31]. Sudden death in post-weaning pigs with or without diarrhea due to endotoxin shock following *Escherichia coli* infection was also reported [27]. Although some serotypes of *E. coli* such as O149 which was regarded as pathogenic have been isolated frequently from the cases of diarrhea, a large number of the other serotypes were also isolated [6, 31]. Subsequently, most of the isolates were found to produce heat-stable toxin (ST) and heat-labile toxin (LT) [31] and were grouped as enterotoxigenic *E. coli* (ETEC) regardless of serotypes. Physiological changes in the intestinal tract caused by stress of weaning, enteric virus infection [3, 13] and waning of passive immunity [6] have been implicated in the occurrence of diarrhea in piglets caused by ETEC during weanling stage.

On PRRS prevalent farms, an increase in secondary bacterial infections was observed [32]. *Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Salmonella* spp, *Actinobacillus pleuropneumonia* and *Mycoplasma hyorhinis* were reported as the microorganisms which caused severe secondary infections [11, 15, 17, 24, 25]. *Escherichia coli* has been regarded as one of the important agents involved in the secondary infection [4].

Increase in severity of illness or increase in the growth of secondary microorganisms were confirmed in pigs experimentally infected with PRRS virus [7, 11, 28]. However, the study of mixed infection involving bacteria and PRRS virus in the field has been hampered due to the difficulties of virus detection. Recently, the polymerase chain reaction (PCR) that detects the PRRS virus at a level of 1 $TCID_{50}$/100 μl was found to be applicable for PRRS virus detection in the field [12]. In this study, we detected PRRS virus by means of the PCR and showed that PRRS virus could be implicated in fatal systemic ETEC infection of weaning pigs.

MATERIALS AND METHODS

Occurrences, clinical signs and samples: In a breeder farm with a productivity of 45,000 piglets a year, an increase of diarrhea in suckling pigs and mortality in weanings were observed from October of 1991. The average mortality per month was 35.6 cases between April to September and it increased to 175 cases from October to March of the next year. The main symptoms of the disease(s) were related to disorders of the alimentary tracts and respiratory organs. Clinical signs of diarrhea in suckling pigs (D pigs), sudden death with diarrhea (SDD pigs) and sudden death without any clinical signs in weaning and post-weaning pigs (SD pigs) were prominent.

Five SDD pigs and 6 SD pigs were examined immediately after death, and 1 pig was examined at moribund (Table 1). The animals were approximately 28 days old in average and were in good nutritional condition. The cerebrum, lung, liver, kidney, mesenteric lymph nodes, heart blood and contents of the small intestine were collected and examined. The samples kept at −80°C were used for virological tests.

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Twelve D pigs of 10 to 14 days old were also examined. Eleven of them recovered from the disease after treatments, but 1 died of emaciation. Rectal swabs were used for tests.

For the purpose of disease monitoring at 1 year after the outbreak, rectal fecese were collected from 9 apparently healthy pigs of age 25 days old. No diarrhea or other diseases were observed before and after the sampling.

Bacteriological examinations

Isolation and titration: Isolation of bacteria was carried out immediately after collection of samples by routine procedures in both Trypticase soy agar plates and deoxycholate hydrogen sulfide lactose (DHL) agar plates. Titration of bacteria was carried out on the supernatant of a 10 percent cerebrum homogenate that had been centrifuged at 500 rpm for 3 min.

Identification of isolates: Biochemical properties of isolates were determined by the API 20E system (API System S.A., Montalieu-Vrcieu, France). The isolates were serotyped by agglutination tests using anti-O and anti-H antisera.

Adherence factor assay: The bacteria were examined for adherence factors K88, K99, and 987P, by the agglutination test using anti-K88, K99, and 987P sera (Denka-Seiken, Tokyo).

Verotoxin (VT) assay: Supernatants of 18-hr broth cultures were clarified through a 450 mμ pore-size filter. The filtrates of test and reference VT1-, VT2- and VTe-positive strains (shown in a following section) were diluted serially and inoculated into Vero cell cultures, as described previously [20]. The cytotoxic effect was regarded as positive when CPE was observed at a dilution of 1:5 or higher within 7 days after inoculation. The filtrates of reference strains showed cytopathic effect (CPE) at a dilution of 1:128 or higher.

Invasiveness assay: Invasiveness of the isolates was tested by plaque formation as described [20]. Briefly, confluent HeLa cell cultures which were grown in antibiotic-free growth medium in 12-well flasks were inoculated with 0.5 m*l* of the serially diluted bacterial suspensions and incubated at 37°C for 90 min. The cultures were then overlaid with growth medium containing 0.5 percent agarose and gentamycin of 20 μg per m*l*. A reference positive strain was always included in the test.

PCR for detection of genes encoding for pathogenicity: DNA isolated from 18-hr broth cultures of test and reference *E. coli* by phenol-chloroform extraction and ethanol precipitation was used as a template for the PCR. The sequences (5'–3') of a pair of oligonucleotide primers used for detection of the heat-labile toxin A subunit (LT-a) [29] were CCGGTATTACAGAAATCTGA and GTGCATGATGAATCCAGGGT, for the heat-stable subunit B (ST-b) [14] of enterotoxigenic *E. coli* were GCAATAAGGTTGAGGTGAT and GCCTGCAGTGAGAAATGGAC, for Verotoxin (VT) 1 [22] were GAAGAGTCCGTGGGATTACG and AGCGATGCAGCTATTAATAA, for VT2 [22] were TTAACCACACCCACGGCAGT and GCTCTGGATGCATCTCTGGT, for VTe [9] were CCTTAACTAAAAAGGAATATA and CTGGTGGTGGTATGATTAATA, and for invasiveness (*inv*) E [8] were ATATCTCTATTTCCAATCGCGT and GATGGCGAGAAATTATATCCCG. The sizes of the amplified products were 110, 377, 130, 346, 230 and 382 base pairs, respectively. Gifu 10659 (WHO 103: ST+), Gifu 10662 (WHO 102: LT), Gifu 12809, Gifu 12810, Gifu 12811 and Gifu 13248 strains were used as the positive reference strains for ST-b, LT-a, VT1, VT2, VTe and *inv*E genes, respectively. The DNA samples (5 μl) were mixed with 45 μl of reaction mixture as described [22]. The mixtures were denatured for 5 min and subjected to 30 cycles of amplification in a DNA thermal cycler (GeneAmp 9600, Perkin Elmer Cetus). Procedures for the amplification cycles were denaturation for 30 sec at 95°C, annealing of primers for 60 sec at 48°C, and primer extension for 90 sec at 74°C with autoextension. The final reaction mixture was analyzed by gel electrophoresis.

Virological examination

Virus isolation: For the isolation of PRRS virus, a 10% lung homogenate was first centrifuged and then clarified through a 450 mμ pore size filter. The filtrate was inoculated into MARC-145 cells [10] which were cultured in 12-well flasks in a CO_2 incubator at 37°C. The cultures were observed for CPE for 9 days following which the culture fluids were subinoculated into newly prepared cell cultures. Virus isolation was regarded as negative if no CPE was observed for 9 days in the subinoculated cultures.

For the isolation of enteric viruses, filtrates of intestinal contents were inoculated into primary porcine kidney cell cultures and observed for CPE for 10 days.

Serological tests: Four litermates from each of the 3 SDD pigs were bled twice at an interval of one month. Sera were also collected from 12 10-day-old pigs showing diarrhea. These sera were tested for antibodies to PRRS virus by the indirect immunofluorescent antibody (IFA) test as described [19] and to transmissible gastroenteritis virus by the neutralization test [26].

Detection of PRRS virus by PCR: SD and SDD pigs were tested. Primer sets and amplification reaction were described previously [12]. Briefly, a sample of RNA to be tested was extracted from filtrates of 10 percent lung homogenates (which were used for virus isolation) by using a commercial RNA isolation kit (RNAzol B, TEL-TEST, Inc., U.S.A.). A common primer set to amplify both the North American type and the European type of PRRS virus was used for reverse transcription (RT)-PCR and 2 specific primer sets to amplify the 2 types of the virus were used for the nested PCR. The amplified products were treated with restriction enzymes, *Pvu*II and *Rsa*I, as described previously [12], to confirm the specificity of the product.

RESULTS

Isolates from SDD and SD pigs: A large number of bacteria of identical appearance were isolated in pure cultures from the cerebrum, kidney, liver, lung, mesenteric

lymph nodes, heart blood and contents of the small intestine on both Trypticase soy agar and DHL agar plates. The number of bacteria per gram of the cerebrum is as shown in Table 1. Each 2 isolates derived from 1 or 2 organs of 11 pigs and 6 isolates derived from cultures of intestinal contents of each 3 SDD and SD pigs were used for the following experiments. All 42 isolates had biochemical properties identical to those of *E. coli.*

In the PCR using primer sets to detect ST-b and LT-a, products which were identical in molecular size to the reference products of ST-b and LT-a were amplified in 40 and 32 of the 42 isolates from the 11 pigs, respectively (Figs. 1 and 2, and Table 1). Thirty isolates produced both products and the remaining 12 isolates produced either one of the two. Each pair of the isolates from an organ showed identical properties (Table 1 and Figs. 1 and 2). On the other hand, no product was amplified when primer sets to detect VT1, VT2, VTe, and *inv*E were used (Table 2 and Fig. 3).

Each strain isolated from the cerebrum of 11 pigs was tested biologically. As shown in Table 2, none of the isolates formed any plaques on Hela cell monolayers. Also, none of the filtrates of the isolates showed any CPE on Vero cells. Three of the 11 isolates were agglutinated by anti-K88 serum but none of them reacted with anti-K99 or anti-987P sera.

Of the 11 strains, 4 were identified as O149: H–, 1 as O149: H10, 4 as O15: H9, 1 as O111: H–, and 1 as OR: H6.

Isolates from D pigs: A large number of *E. coli* were also isolated from the rectal swabs of 12 D pigs. An isolate from each of the 12 pigs was taken for experiments. In the PCR, all of the isolates were shown to carry a LT-a gene and 10 out of 12 carried a ST-b gene. Verotoxin genes and *inv*E gene were not detected (Table 2).

Isolates from healthy pigs: A small number of *E. coli* were isolated from the rectal feces of 9 healthy pigs. An isolate from each of the 9 pigs was used for experiments. One isolate was both LT-a and ST-b positive, 2 other isolates were ST-b positive, and the other 6 isolates were both negative. Verotoxin genes and *inv*E gene were not detected (Table 2).

Detection of PRRS virus: No CPE agent was isolated in MARC-145 cell cultures after the primary and subinoculation of the lung homogenates from 11 pigs. In the nested PCR using the product of RT-PCR and the specific primer set for North American type virus a product identical in size (349 bp) to that for PRRS virus was amplified in all cases (Fig. 4). However, the product was not detected in the nested PCR that used a specific primer set for European type virus. Some of the amplified products were treated with 2 restriction enzymes. As shown in Fig. 5, *Pvu*II digested the product but *Rsa*I did not. Similar digestion result were obtained with North American type PRRS virus.

Detection of other CPE agents: No CPE was observed in porcine kidney cell cultures after inoculation with 11 filtrates from the intestinal contents of SDD and SD pigs.

Serological tests: Sera collected from 12 10-day-old pigs had IFA antibody titers of 320 to 1,280 against PRRS virus. In 12 weaning pigs, IFA antibody titers were between < 40 and 160. Seven of them showed 4-fold or more increase in titer after one month. No antibody against TGE virus was detected.

DISCUSSION

On the farm where an increase in fatal cases was observed, prevalence of diarrhea in suckling and weaning pigs was recognized. Although some of the suckling pigs died of dehydration and emaciation, most of them recovered after medical treatment. In contrast, occurrences of sudden death after short duration of diarrhea and sudden death without any signs were one of the prominent characteristics of the disease in weaning pigs. Involvement of TGE virus and other enteric viruses in the diarrhea was ruled out but presence of PRRS virus was confirmed by PCR and serological studies.

Septicemia caused by *E. coli* was regarded as the primary cause of the sudden death since large numbers of *E. coli* were detected from all the tissues tested including the cerebrum and heart blood. It is likely that the bacteria had multiplied in the tissues or in the blood before the death of the piglets because the tissues were cultured bacteriologically within 1 hr after death. Recovery of *E. coli* in the same condition from the pig in moribund supported this view.

Large numbers of *E. coli* were also found in rectal feces of D pigs. All of the *E. coli* derived from SDD, SD and D pigs were ETEC carrying both LT-a and ST-b, or LT-a or ST-b. The ETEC did not have the enteropathogenic genes such as VT1, VT2, VTe and the invasive gene (*inv*E). The results suggested that the pathogenicity shown by the ETEC was related to the LT-a and ST-b genes, although the investigation of other pathogenic genes relating to hemolysis, adherence and invasiveness may be required in the future.

Among the 11 isolates from the 11 SDD and SD pigs, 5 and 4 isolates belonged to the serotypes O149 and O15. The former has often been isolated from cases of diarrhea and regarded as pathogenic [6, 31] while the latter was isolated only in rare cases [6]. Furthermore, one isolate was identified as the rough type which is generally known to be avirulent. Therefore, relationship between serotype and pathogenicity should be discussed after further experiments.

PRRS virus infection was demonstrated in the lungs of every SD and SDD pig. The virus infection was characterized by reproductive failure and respiratory disease [4, 5]. However, on a farm contaminated persistently with PRRS virus, subclinical infection is known to be another important feature of the disease [4, 5, 23]. On such farms, prevalence of the virus was rare among suckling pigs due to the presence of colostral antibodies [16], while it was active among weaning pigs due to the decrease of the antibodies

Table 1. Isolation of *E. coli* from pigs that died suddenly with diarrhea (SDD) and those that died suddenly without signs (SD) and detection of enterotoxigenic genes from the isolates

Individual	Age (Days)	Symptom	Tissue and colony examined	No. of *E. coli* per gram the cerebrum	Enterotoxine gene LT-a	ST-b
A	25	SD	Cerebrum-1[a)]	2.7×10^8	–	+
			-2		–	+
			Kidney-1		–	+
			-2		–	+
B	36	SDD	Cerebrum-1	4.3×10^8	–	+
			-2		–	+
			Intestine[b)]-1		+	+
C	20	SDD	Cerebrum-1	1.4×10^8	+	+
			-2		+	+
			Lymph node-1		+	+
			-2		+	+
D	40	SD	Cerebrum-1	1.2×10^4	+	+
			-2		+	+
E	26	SDD[c)]	Cerebrum-1	1.0×10^2	+	–
			-2		+	–
F	36	SD	Cerebrum-1	5.0×10^1	+	+
			-2		+	+
			Kidney-1		+	+
			-2		+	+
			Intestine-1		+	+
G	25	SD	Cerebrum-1	2.6×10^2	+	+
			-2		+	+
			Kidney-1		+	+
			-2		+	+
			Intestine-1		+	+
H	25	SD	Cerebrum-1	3.7×10^3	+	+
			-2		+	+
			Kidney-1		+	+
			-2		+	+
			Intestine-1		+	+
I	25	SD	Cerebrum-1	1.0×10^7	–	+
			-2		–	+
			Kidney-1		–	+
			-2		–	+
			Intestine-1		+	+
J	25	SDD	Cerebrum-1	7.0×10^1	+	+
			-2		+	+
			Intestine-1		+	+
K	25	SDD	Cerebrum-1	5.0×10^1	+	+
			-2		+	+
			Lymph node-1		+	+
			-2		+	+

Among total 42 strains tested, 32 were LT+, 40 ST+, and 30 both positive.

a) Each 2 colony derived from one organ was tested. b) Intestinal contents. Each one colony was tested. c) The pig was tested at moribund.

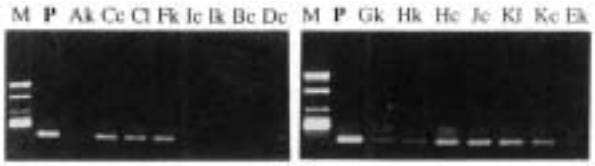

Fig. 1. (left and right). Detection of an enterotoxin gene, LT-a, in *E. coli* isolated from SD and SDD pigs by PCR. Results of each 1 strain from 7 pigs and each 2 strain from 4 pigs are shown. Code of an isolate: the first capital letter identifies an individual pig and the 2nd small letter indicates the tissue from which the strain was isolated (c: cerebrum, k: kidney, l: mesenteric lymph node). M: marker (each band shows 1,327, 907, 519 and 341 bp) P: positive reference strain.

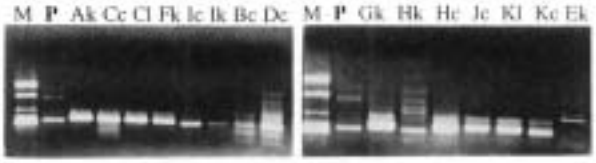

Fig. 2. (left and right). Detection of an enterotoxin gene, ST-b, in *E. coli* isolated from SD and SDD pigs by PCR. Results of each 1 strain from 7 pigs and each 2 strain from 4 pigs are shown. See the legend for Fig. 1.

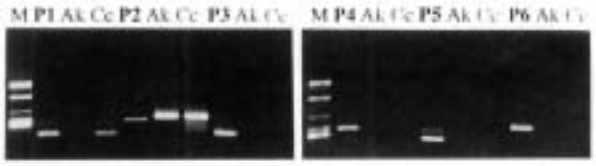

Fig. 3. (left and right). PCR for detection of Verotoxin genes and an invasive gene. Results using reference positive strains (shown as P1-P6) and 2 isolates (Ak and Cc) are shown as examples. Detection of LT-a and ST-b is also shown. P1: LT, P2: ST, P3: VT1, P4: VT2, P5: VTe, P6: *inv*E, and M: marker. See legend for Fig. 1.

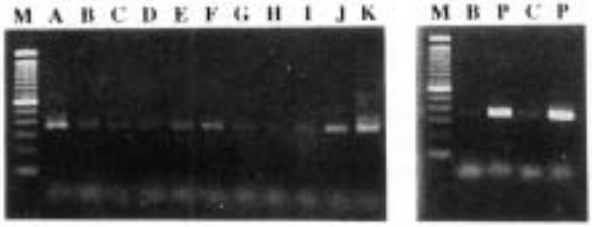

Fig. 4. (left and right). Detection of cDNA of PRRS virus in the lung homogenates of 11 SD and SDD pigs (shown as A-K) by the nested PCR. Specific primers for North American type PRRS virus (12) was used. The size of the products (349 bp) was equal to that of a positive reference strain (shown as P). M: marker (100 bp ladder).

Fig. 5. Digestion of the nested PCR products derived from the lung of a SD pig "K" and a reference PRRS virus strain, EDRD, with restriction enzymes *Pvu*II (P) and *Rsa*I (R). C: control (a dilution buffer of restriction enzymes). M: marker.

Table 2. Pathogenic properties of *E. coli* isolated from SD and SDD pigs, suckling pigs that showed diarrhea (D), and weaning healthy (H) pigs

Pathogenic factor		SD and SDD pig: Biological test	SD and SDD pig: Serological test	SD and SDD pig: PCR	D pig: PCR	H pig: PCR
Invasiveness	*inv*E	0/11[a)]	NT	0/42	0/12	0/9
Entero-pathogenicity (Verotoxin)	VT1	0/11	NT	0/42	0/12	0/9
	VT2	0/11	NT	0/42	0/12	0/9
	VTe	0/11	NT	0/42	0/12	0/9
Exterotoxin	LT	NT	NT	32/42	12/12	1/9
	ST	NT	NT	40/42	10/12	3/9
Adherence factor	K88	NT	3/11	NT	NT	NT
	K99	NT	0/11	NT	NT	NT
	987P	NT	0/11	NT	NT	NT

a) Positive number of colony/Number of colony tested. NT: Not tested.

[1]. Similarly, on the farm studied, passive antibodies were detected in suckling pigs, and PRRS virus infection was found in the weaning pigs.

It seemed that sudden death in weaning pigs caused by ETEC septicemia was a result of concurrent infections with ETEC and PRRS virus, the both being activated following the decline of passive immunity to the agents [1, 6]. PRRS virus does have a distinct affinity for lung macrophages [21, 30], kills a high percent of these cells [33], and impairs lung-level and systemic-level immune defenses [4, 16, 18]. These effects might assist the "*in vivo*" invasion and replication of ETEC in the extraintestinal sites.

On the other hand, in the suckling pigs, PRRS virus infection may have been prevented because of the effect of passive immunity as shown previously [16]. It was assumed that, although ETEC caused diarrhea by replication in the intestinal tract, the "*in vivo*" invasion of ETEC was prevented by host defense mechanisms. It was therefore hypothesized that sudden death in weaning pigs was caused by a combination of at least 3 factors: first, an age factor relating to decline of passive immunity; second, infection by PRRS virus which reduces the resistance to bacterial infections; third, "*in vivo*" replication of ETEC which leads the host to death.

In this study, a small number of ETEC which had similar properties with those isolated during the endemic stage of diarrhea and sudden death, were isolated from the rectal feces of healthy weaning pigs in the quiescent stage on the farm. Thus, it was shown that the growth of the ETEC isolated in the study herein was limited in the healthy intestinal tract, but the organisms persisted in the individual animals on the farm. Diseases outbreak occurred when the pigs were exposed to certain factors such as virus infection [3, 16] or stress which broke down the normal intestinal environments. PRRS virus infection may be one of the most important factors contributing to disease outbreak. In this study, PRRS virus was detected only by the PCR but was not isolated in cell cultures. The reason for the failure of virus isolation was not apparent but low sensitivity of MARC-145 cells to the virus [2, 10], may be one of the reasons. The infecting virus was, however, identified as the North American type virus because the amplification was observed only by the use of the specific primer set that amplified only cDNA of North American type virus by the nested PCR [12]. Further analysis of the PCR products by the restriction enzymes [12] suggested that the products had nucleotide arrangements that were similar to those of the North American type virus.

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