HEMAGGLUTINATION AND COLONY HYBRIDIZATION FOR THE IDENTIFICATION OF ENTEROTOXIGENIC *Escherichia coli* ISOLATED FROM HEALTHY PIG

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Summary

Erythrocytes from three different animal species were used to determine mannose-sensitive hemagglutination (MSHA) and mannose-resistant hemagglutination (MRHA) of 755 isolates obtained from rectal swabs of healthy pig. In addition, colony hybridization using digoxigenin-dUTP labeled polynucleotide probes was performed for the detection of heat-stable and heat-labile enterotoxin genes carried by MRHA positive isolates. Of 755 strains, 9, 4, and 28 strains gave a positive MRHA with bovine, equine, and pig erythrocytes, respectively. Of these isolates, 28 (3.7%) were characterized for positive MRHA by at least one blood. Seven isolates gave a positive MRHA with two kinds of blood. Three gave a positive MRHA with three kinds of blood. Twenty-eight strains, while positive in MRHA, yielded negative signals in the colony hybridization assay for the detection of heat-stable (STaI and STaII) and heat-labile (LT) enterotoxin genes in *E. coli*.

(**Key Words** : MSHA, MRHA, Colony Hybridization, Enterotoxigenic *E. coli*)

Introduction

Enterotoxigenic strains of *Escherichia coli* (ETEC) which may be pathogenic for human and young animals, produce plasmid-mediated enterotoxins (Dallas et al., 1979, Mosely et al., 1983). Enterotoxins produced by these organisms are classified into two main groups, heat-stable toxins (ST) and heat-labile toxins (LT). The ST includes a methanol-soluble STa (STI) and a methanol insoluble STb (STII) (Burgess et al., 1978). The STa consists of two chemically similar toxins, the STaI (STp) and the StaII (STh). STaI is produced by both human and pocine isolates of ETEC, whereas STaII is produced by human isolates only (Mosely et al., 1983, Yamamoto et al., 1987). The STb is rarely produced by only ETEC strains isolated from humans (Echeverria et al., 1985). The heat-labile (LT) toxin is immunologically similar to cholera toxin and, like cholera toxin, exerts its effects by stimulation of adenylate cyclase within the epithelial cells of the upper intestine (Gyles, 1974). Restriction fragments of the insert-containing portions of the LT gene have been observed to hybridize with *Vibrio cholerae* DNA (Dallas and Falkow, 1979). Traditionally, STaI and STaII have been detected by the suckling mouse assay (Dean et al., 1972), whereas the STb toxin is detected in ligated jejunal loops of pigs (Burgess et al., 1978). Numerous methods for ETEC detection have been described in recent years. Detection of the plasmid-borne toxin genes using gene probes have proven most valid (Sommerfelt et al., 1988a). Restriction endonuclease-generated DNA fragments harboring specific toxin gene sequences, as well as synthetically produced oligonucleotides constructed to match such gene, have been used as probes for the identification of ETEC. In general, compared with the standard bioassays for detecting the phenotypic expression of these genes, the efficiency of colony hybridization with these two classes of probes has showed a perfect concordance in their specific detection and differentiation of enterotoxigenic *E. coli*. The signal strength on autoradiography after hybridization with oligonucleotides was weaker than that obtained after hybridization with polynucleotide probes (Sommerfelt et al., 1988_b).

Another virulence factor associated with enterotoxigenic *E. coli* strains is their ability to attach to mucosal surface. Pili (fimbriae) enable *E. coli* strains to adhere to the gut epithelium. *E. coli* strains isolated from piglets with diarrhea may possess K88, 987P fimbrial antigens; seldom occur K99, F41, and F17. Many strains

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isolated both from diarrheal and healthy piglets possess type 1 fimbriae (F1) (Osek and Truszczynski, 1992). *E. coli* that synthesize K88 antigen caused mannose-resistant hemagglutination (MRHA) of guinea-pig erythrocytes in a microhemagglutination test (Jones and Rutter, 1974). ETEC strains of serotypes O7, O15, O25, O115 and O128 gave MRHA of human or calf erythrocytes (Cravioto et al., 1982). ETEC strains isolated from human with diarrhea had two fimbriate surface factors known as colonization factor antigens (CFA) I and II (Evans and Evans, 1978, Evans et al., 1975). CFA/I causes MRHA of human and bovine erythrocytes (Evans et al., 1977) and is controlled by a plasmid which also codes for the production of heat-stable enterotoxin (McConnell et al., 1981, Reis et al., 1980).

The present study examined hemagglutinating fimbriae and enterotoxin genes in *E. coli* strains isolated from healthy pig groups dieted competitive exclusion (CE) product.

Materials and Methods

Experimental group

One hundred weanling pigs were randomly divided into two groups (50 pigs/group). Each group was subdivided into 10 pens of 5 pigs. One group served as the control. The second group was fed a commercial mannanoligosaccharide competitive exclusion (CE) product (Bio Mos; Alltech) at the ratio of 0.907 kg/907 kg of diet.

Bacterial strains

All pigs were sampled in the morning that feeding of the CE product began (A group), after feeding of the CE product for 1 month (B group) and when the pigs reached market weight (C group). Rectal swab obtained from each pig were streaked on MacConkey agar plate, after incubation at 35℃ for 18 hr, up to 25 lactose-positive isolates. Isolates were picked to trypticase soy agar deeps, incubated, and placed at 2℃ until used. The 755 isolates used in this study represented every fifth isolate obtained from MacConkey agar. ATCC strains containing ST and LT genes (table 1) were used as controls for the probes.

TABLE 1. *E. coli* STRAINS USED IN THIS STUDY

Strains	Serotype	Type of toxin production	Source	Gene location	Reference
ATCC 43896	O78:K80:H12	STaII	Human	Plasmid	Sommerfelt et al., 1988$_b$
ATCC 35401	O78:H11	STaI, STaII, LT	Human	Plasmid	Evans et al., 1975
ATCC 43886	O25:K98:NM	LT	Human	Plasmid	Söderlind and Möllby, 1979

Microhemagglutination test

Bovine, equine and pig bloods were aseptically drawn and placed into sterile flasks containing 10 ml of sterile 3.8% citric acid per 90 ml of blood. The erythrocytes were collected by centrifuging and diluted with 0.1 M phosphate-buffered saline (PBS; 0.85% NaCl, pH 7.2 with and without 1% D-mannose) to make a 2% erythrocyte cell suspension of packed cells. Bacterial cells were harvested with PBS from cultures grown on CFA agar (Evans et al., 1977) for 20 hr at 37℃ and adjusted to an optical density (640 nm) of approximately 2.0. All the *E. coli* (775 strains) isolated from healthy pig were prepared as test sample. Serial two-fold dilutions of each bacterial suspension (50 μl) were prepared in the wells of microtiter plates (Falcon 3911, Becton Dickinson Co. U.S. A.) and equal volumes of 2% erythrocyte suspension were added. Microtiter plates were rotated at 150 rpm for 20 min. and kept at 4℃ for 3-4 hr when the degree of agglutination was determined visually. Agglutination of erythrocytes in dilutions of bacterial suspension above 1:8 was considered positive (Osek and Truszczynski, 1992).

Probe preparation

Plasmid DNA was purified by the method of Sambrook et al. (1989). To obtain pDAS100, pDAS101, and pEWD299, LB agar containing ampicillin at concentration of 50 μg/ml was used as a bacterial culture medium. The enzyme-generated polynucleotide probe were prepared by using pDAS100, pDAS101, and pEWD299 which construct STaII, STaI, and LT gene, respectively. The STaII insert was cleaved from pDAS 100 using *Bam*HI and *Hind*III (Sommerfelt et al., 1990). STaI gene was obtained from pDAS 101 using *Eco*RI and *Bam*HI, and LT was taken from pEWD299 using *Hin*dIII and *Eco*RI (Sommerfelt et al., 1988$_b$). After digestion, fragments obtained after electrophoretic separation in 1.5% agarose were isolated with the Geneclean Kit(Bio 101 Inc., La Jolla, Calf. U.S.A.) according to the instructions of the manufacturer for use as probes. For the nonradioactive hybridization assay, the polynucleotide

fragment were labeled by random hexanucleotide-primed, Klenow enzyme-mediated incorporation of digoxigenin-labeled dUTP, as recommended by the kit manufacturer (Genius kit; Boehringer Mannheim). The labeled, denatured probes were added to a final concentration of 8 ng/ml in hybridization solution.

Colony Hybridizations

180 strains (155 positive strains by at least 1 blood with MRHA test in dilution of bacterial suspension above 1:1, and 25 negative strains as a control) were spotted on LB agar plate and grown at 37℃ for 20 hr. A modification of the detection procedure outlined in the Genius System User's Guide for Filter Hybridization (Boehringer Mannheim) was used for clony hybridzation, with a alteration in the baking condition of nylon membranes. Thus, replicas of bacterial spotted plates were transferred onto nylon supports by placing the membranes directly onto the surface of the bacterial colonies on LB agar plate. After the nylon transfer membranes were denatured with lysis solution (0.5M NaOH, 1.5M NaCl) and neutralized, they were baking in a vacuum oven at 120℃ for 30 min to fix the DNA to the membrane. To minimize health hazards, formamide was eliminated from the prehybridization and hybridization mixtures. Prehybridization was performed at 65℃ for 2-4 hr, and hybridization was performed overnight at 65℃. The washing conditions described by Sommerfelt et al. (1990) was used, that is, the washing solution of STaII, STaI and LT probes used was 3 × SSC (1 × SSC is 0.15M sodium chloride plus 0.015M sodium citrate with 0.1% sodium dodecyl sulfate), 1 × SSC, and 0.2 × SSC, respectively, with a constant temperature of 65℃ for 1-3 min. After wasing, the hybridized digoxigenin-labeled probes were combined by anti-digoxigenin-alkaline phosphatase immunologically. Antibody hapten complex was detected by 5-brome-4-chloro-3-indolyl phosphate toluidinium salt and nitroblue tetrazolium salt to purple-blue color.

Results

Hemagglutination (HA) distribution in bacterial cell dilution with bovine, equine, and pig erythrocytes was shown in table 2. After one month of CE treatment (B group), mannose-sensitive HA (MSHA) strains with bovine and equine erythrocytes were decreased as compared with control group and, then at market weight pig (C group), MSHA positive strains were increased. MRHA positive strains were increased after CE treatment. Of the 755 *E. coli* strains, nine isolates gave a positive MRHA with bovine erythrocytes, four with horse erythrocytes, and twenty-eight with pig erythrocytes. The

TABLE 2. DISTRIBUTION OF MANNOSE-SENSITIVE HA AND MANNOSE-RESISTANT HA POSITIVE *E. coli* STRAINS IN PIGS

Source of *E. coli*	No. of strain tested	No (%) of MSHA* strain with erythrocytes from:			No (%) of MRHA** strain with erythrocytes from:		
		bovine	equine	pig	bovine	equine	pig
Before treatment (A group)							
Control	107	0	4 (3.7)	104 (97.1)	3 (2.8)	0	3 (2.8)
CE-treated	121	0	3 (2.5)	119 (98.3)	2 (1.7)	0	2 (1.7)
One month of treatment (B group)							
Control	139	1 (0.7)	15 (10.8)	133 (95.7)	0	1 (0.7)	6 (4.3)
CE-treated	133	0	3 (2.3)	127 (95.5)	3 (2.3)	3 (2.3)	6 (4.5)
At market weight (C group)							
Control	120	0	4 (3.3)	117 (97.5)	1 (0.8)	0	3 (2.5)
CE-treated	135	3 (2.2)	7 (5.2)	127 (94.1)	0	0	8 (5.9)
Total	755	4 (0.5)	36 (4.7)	727 (96.3)	9 (1.2)	4 (0.5)	28 (3.7)

Mannose-sensitive HA*: Agglutination of red blood cells was occurred in dilution of bacterial suspension above 1:8, whereas that reaction with 1% mannose was negative.

Mannose-resisant HA**: Agglutination of red blood cells containing 1% mannose was occurred in dilution of bacterial suspension above 1:8.

ratios of MRHA and MSHA positive with bovine, equine, and pig erythrocytes were 2.2, 0.11, and 0.04, respectively. Pig erythrocytes had a 100% HA positive suggesting that surface structure of pig erythrocytes may have agglutinated with *E. coli* cells well. Of the 228 *E. coli* strains isolated from the piglets (A group), 5 strains (2%) gave positive MRHA with bovine and pig erythrocytes. Of the 272 *E. coli* strains isolated from the pigs after 1 month feeding (B group), 12 strains (4%) gave positive MRHA with pig erythrocytes. Of the 255 *E. coli* strains isolated from the market weight pigs (C group), 11 strains (4%) gave positive MRHA with pig erythrocytes (table 2). Twenty-eight strains showed MRHA positive by at least one blood. Seven strains gave a positive MRHA with two kinds of erythrocytes. Three strains did with three kinds of erythrocytes. Sixty % of 28 strains had plasmid. All of 9 strains which gave positive MRHA with bovine erythrocytes had plasmid (table 3).

Three kinds of plasmid DNA fragments generated by restriction enzyme were used as probes (figure 1).

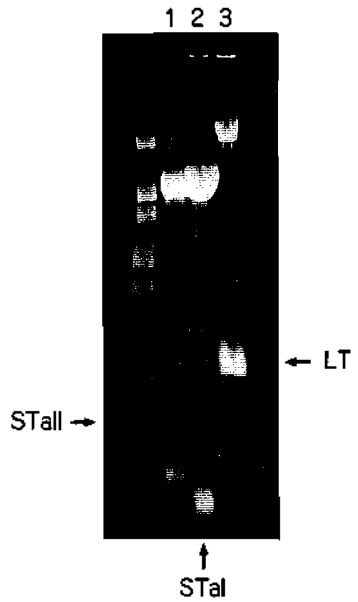


Figure 1. Restriction enzyme-generated STaII (lane 1), STaI (lane 2), and ST (lane 3) gene fragments of pDAS100, pDAS101, and pEWD299, respectively, separated on ethidium bromide stained 1.5% agarose gel. pDAS100 was digested with *Bam*HI and *Hin*dIII, pDAS101 was digested with *Eco*RI and *Bam*HI, and pEWD299 was digested with *Hin*dIII and *Eco*RI. Size markers contain 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, 154 bp DNA fragments.

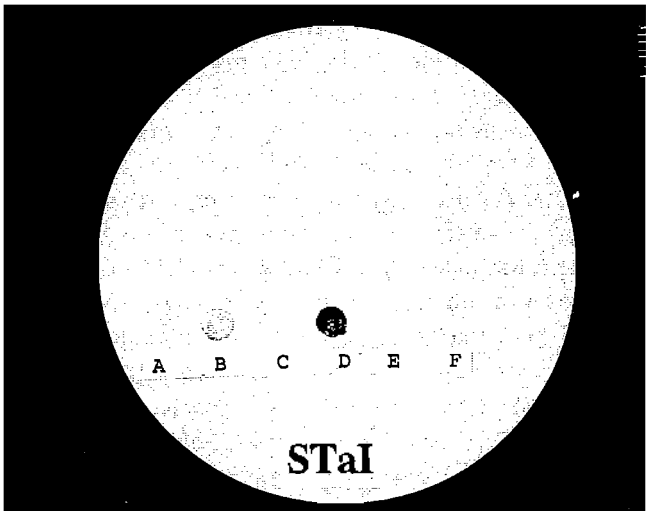
Digoxigenin-dUTP labeled ETEC probes specifically identified strains known to harbor genes coding for heat-stable (ST) enterotoxins and heat-labile (LT) toxins (figure 2). Probes differentiated STaI, STaII, and LT producing *E. coli* strains (ATCC43896, ATCC35401, ATCC43886). True positive reactions had the typically deeper-stained purple-blue of nitroblue tetrazolium and 5-bromo-4-chloro-

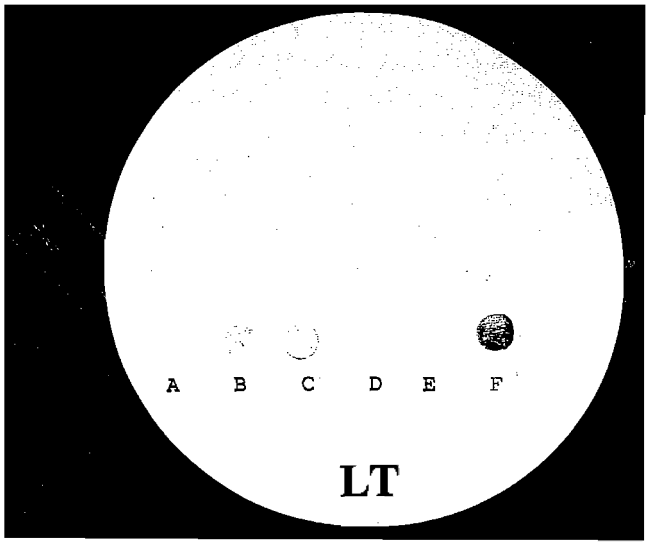
TABLE 3. MRHA PATTERN AND PLASMID PRESENCE IN *E. coli* STRAINS ISOLATED FROM PIG GROUPS

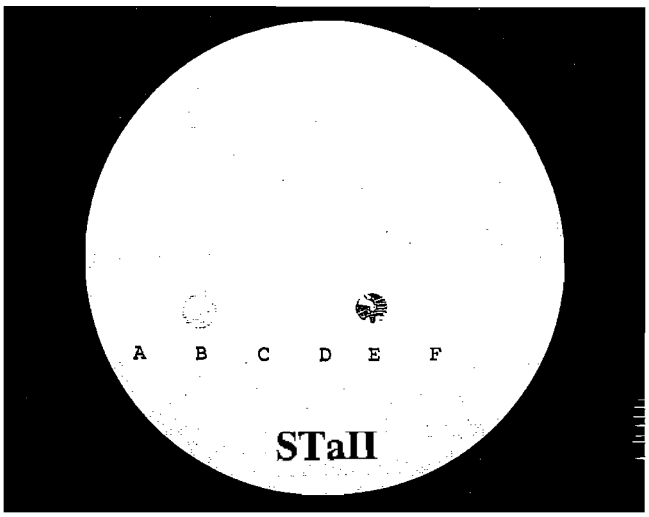
E. coli strains isolated from pig feces	Source of *E. coli*	Indicated MRHA pattern*			Plasmid presence
		bovine	equine	pig	
A8	1	+		+	+
A9	1	+		+	+
A11	1	+		+	+
A143	2	+		+	+
A145	2	+		+	+
B2	1		+	+	
B180	1			+	
B181	1			+	
B218	1			+	+
B221	1			+	+
B251	1			+	
B126	2	+	+	+	+
B142	2			+	
B143	2			+	+
B144	2	+	+	+	+
B191	2	+	+	+	+
B273	2			+	+
C124	1	+		+	+
C181	1			+	+
C225	1			+	+
C49	2			+	+
C50	2			+	
C75	2			+	
C76	2			+	+
C87	2			+	
C88	2			+	
C153	2			+	
C231	2			+	

A: isolate from A group, B: isolate from B group, C: isolate from C group, 1: *E. coli* isolated from control pig groups, 2: *E. coli* isolated from diet pig groups.

* : Agglutination of erythrocytes containing 1% mannose was occured in dilution of bacterial suspension above 1:8.







A : ATCC 43896; produces STaII enterotoxin.
B : ATCC 35401 ; produces LT, STaI, and STaII enterotoxin.
C : ATCC 43886; produces LT enterotoxin.
D : The *E. coli* strain harboring the pDAS101 of the STaI gene (Sommerfelt et al., 1988_b).
E : The *E. coli* strain harboring the pDAS100 of the STaII gene (Sommerfelt et al., 1990).
F : The *E. coli* strain harboring the pEWD299 of the LT gene (Sommerfelt et al., 1988_b).

In all three assays, all strains isolated from pig feces were identified as non-enterotoxigenic *E. coli*, whereas ATCC 43896 was identified as $STaII^+LT^-$, ATCC 35401 was $STaI^+STaII^+LT^+$, ATCC 43886 was ST^-LT^+.

Figure 2. Results of colony hybridization with digoxigenin-dUTP labeled polynucleotide probe of STaI, STaII and LT gene. We examined *E. coli* strains had MRHA along with six control colonies.

3-indolyl phosphate toluidinium salt. On the other hand, the lysed colonies of toxin-negative strains had a yellowish color. This information proved to be essential when registering the results of the nylon membrane. Very little background color was discernible on the membrance. Analyses of the 180 strains (155 positive strains by at least 1 blood with MRHA test in dilution of bacterial suspension above 1:1, and 25 negative strains) were all negative in the three kinds of toxin gene colony hybridization assay, whereas ATCC43896 was identified as $STaII^+LT^-$, ATCC35401 was $STaI^+STaII^+LT^+$, ATCC43886 was ST^-LT^+ in the nylon membranes. Similar hybridization patterns were seen when isolated plasmids from the three ATCC strains were spotted directly on to the nylon membranes. These results verified the existence of the enterotoxin genes on plasmids from known enterotoxigenic strains.

Discussion

The fimbriae with MS adhesion were classified as 'type 1'. A possible explanation of the adhesive property of type 1 fimbriae is suggested by the observations that the electrophoretic mobility of the fimbriate *E. coli*. The fimbriate bacteria must therefore have a lower density of negative surface charge and a more hydrophobic character than the non-fimbriate bacteria, and these properties will facilitate their close approach to the negatively charged, hydrophilic surfaces of erythrocytes (Duguid and Old, 1980). The simple explanation of the inhibitory action of D-mannose is that it serves as a soluble analogue of fixed D-mannose-like residues on the surface of erythrocytes,

and blocks adhesive sites on the fimbriae that otherwise would bind to the residues on the cell (Ofek et al., 1977). Bacteria have lectins on the cell surface that recognize specific sugars and allow the cell to attach to that sugar. These sugars can be found on the epithelial cell surface. Binding of bacteria has been shown to be mediated by a mannose-specific lectin-like substance on the bacterial cell surface. If dietary mannan-oligosaccharides can occupy potential bacterial binding sites on intestinal epithelium, then colonization may be precluded. Alternatively, mannans may bind bacterial cell surface sites and pass throught the digestive tract without harming the host. These microorganisms have type 1 fimbriae, which adhere to mannose sugars or mannan chains (Lyons and Jacques, 1994). We found that after one month of CE treatment, MSHA positive strains with bovine and equine erythrocytes were decreased as compared with control group (table 2). It was suggesting that CE product diet could reduce MSHA strains from intestinal track of CE dieted pig.

The positive MRHA was noted in 72.8 and 53.0% of strains isolated from suckling and weaned piglets with diarrhea, respectively. Only 5 strains (0.7%) isolated from healthy piglets possessed the positive MRHA. Many strains isolated from healthy piglets, which possessed almost exclusively F1 fimbriae, in most cases were non-enterotoxigenic (Osek and Truszczynski, 1992). Mannose-resistant HA is anion vitro model for adhesiveness of ETEC. Interestingly, K88, K99, and CFA demonstrate specificity as hemagglutinins, apparently reflecting the fact that each of these antigens demonstrates host specificity in adhering to intestinal epithelial cells (Evans et al., 1977). A total of 149 nontoxigenic strains of serogroups O6, O8, O63, and O78 were also examined, and only 33 (22%) of the strains gave MRHA with human and calf erythrocytes; 2 (1%) of the strains with calf erythrocytes. None of these strains possessed CFA/I or CFA/II, as determined by immunodiffusion testing (Cravioto et al., 1982). The frequency of isolation of strains with K88 antigen was 56 % in piglets with diarrhea, while this adhesion factor was found only in 1% of the healthy piglets. LT produced by strains isolated from 57% of the diseased piglets, while the corresponding numbers for the two groups of healthy piglets were 5 and 3%, respectively (Söderlind and Möllby, 1979). Elaboration of ST and LT by cultures of *E. coli* is mediated by genes carried on transferable plasmids. In other for these two toxins to function in the small intestine, colonization factors or adhesions must also be produced by the cultures (William, 1986). These factors allow the bacteria to adhere to the mucosa where they grow and elaborate their toxins (Wachsmuth, 1984). It was found out that the ratios of MRHA and MSHA positive with bovine, equine and pig erythrocytes were 2.2, 0.11 and 0.04, respectively (table 2). All the MRHA positive strains from piglet sample (A group) and the plasmid carrying strains were detected by bovine erythrocyte (table 3), therefore bovine erythrocyte was the most effective for identifying MRHA using microhemagglutination test. Nine strains (1.2%) of MRHA positive with bovine erythrocytes among 775 *E. coli* isolated from healthy pig may have colonization factor of fimbriae controlled by plasmids. In this respect, Osek and Truszczynski (1992) stated that the positive MRHA was noted in 0.7% of strains isolated from healthy piglets.

Probes labeled with digoxigenin and stored at −20℃ appeared stable for longer than 6 months. Experiments in our laboratory showed that hybridization solutions containing digoxigenin-labeled probes could be stored at −20℃ after use and reutilized up to four times without significant decrease in sensitivity. Digoxigenin-labeled probes of plasmid DNA generates was suitable for economical nonradioactive labeling and detection of ETEC. Our experience indicates that 28 positive MRHA strains (3.7%) among 755 *E. coli* isolated from 100 herds of healthy pigs yielded negative signals in the colony hybridization assay (figure 2). We must deal with the question of colonization by *E. coil* that do not produce enterotoxin. There is also the posibility that some strains of MRHA-positive *E. coli* may reveal enterotoxin activities other than those currently identifiable.

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