DEVELOPMENT AND EVALUATION OF IMMUNOCHROMATOGRAPHIC ASSAY FOR SIMPLE RAPID DETECTION OF *ESCHERICHIA COLI* O157, O26, O111 AND VERO TOXIN 1/ 2 IN FOOD PRODUCTS

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***Abstract*—**NH Immunochromato (NH IC) O157, O26, O111 and VT1/2 kits were developed as immunochromatographic tests for the confirmation of *Escherichia coli* (*E. coli*) O157, *E. coli* O26, *E. coli* O111 and vero toxin (VT) 1/2, respectively, in food products. NH IC VT1/2 allows detection of both VT1-positive and VT2-positive samples individually with the same device. The lowest detectable amount of *E. coli* O157, *E. coli* O26 and *E. coli* O111-strains in a cell suspension is 10^4 CFU/ml. The lower detection limits for VT1 and VT2 are 2.5 and 1.25 ng/ml, respectively. As to specificities, NH IC O157, O26, O111 and VT1/2 all yielded negative results in this study. To evaluate the ability of NH IC tests to detect their targets in food products, suspensions from supermarkets inoculated with *E. coli* strains, then 10-fold-serial-diluted, were tested with the NH IC kits. The results showed that if target inoculation exceeds 10^0 CFU per 25 mg of food, the NH IC yields positive results. The NH IC tests were simple to perform and achieved detection of their targets, specifically in food, within 15 min. These results suggest that the NH IC device has sufficient sensitivity and specificity, suggesting the usefulness of this simple and rapid method of testing food.

Index Terms*—Escherichia coli*, food, immunochromatography, vero toxin.

I. INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) strains have emerged as an important cause of food-borne infections. They are associated with sporadic outbreaks of, for example, diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Karmali., 1989; Griffin et al., 1991; Nataro et al., 1998; Paton et al., 1998)). Although *E. coli* O157:H7, a prototype of EHEC, has been associated with most important food-borne outbreaks in the United States (Wells et al., 1983) and Canada (Johnson et al., 1983), other EHEC serotypes, particularly O26 and O111, have also emerged as significant causes of human diseases over the past 25 years (Caprioli et al., 1997; Johnson at al., 1996; Tarr at al., 1996). The morbidity and mortality associated with EHEC infections have highlighted the need to develop simple rapid EHEC screening methods for foods.

In Japan, food-borne outbreaks related to EHEC have been traced to O157 (65%), O26 (21%), O111 (4%) and other strains. Therefore, the Ministry of Health, Labour and Welfare announced that a 'Laboratory procedure for enterohemorrhagic *Escherichia coli* O157 and O26 in food' and that the detection of *E. coli* O157, *E. coli* O26 and vero toxin are necessary. Although the methods currently used in health sciences institutes, such as MacConkey agar, are reliable, they require skilled personnel and a well-equipped laboratory. In addition, the procedures are time-consuming, taking at least 4 days, underscoring the need for a simple rapid non-culture assay for the detection of EHEC in food. Immunochromatographic tests are known to be both rapid and simple to performe. Therefore, we developed NH Immunochromato O157, O26, O111 and VT1/2 tests.

II. MATERIALS AND METHODS

Bacterial strains and culture conditions. In total, 23 bacterial strains representing *E. coli* and other bacterial species were used in this study (Table 2). These strains were obtained from the American Type Culture Collection (ATCC), Research Institute for Microbial Diseases, Osaka University (RIMD), Japan Collection of Microorganisms, RIKEN BioResource Center (JCM) and the Institute of Medical Science, The University of Tokyo (IID). The bacterial strains were propagated in Trypto-Soya Broth (TSB) (Nissui Pharmaceutical., Tokyo, Japan) and maintained at the Micro Bank (Iwaki). Liquid cultures were obtained by growing bacteria in mEC-broth with Novobiocin (NmEC) (Merck) for 22 h at 42℃ and in TSB for 22 h at 37℃ in a circulating air incubator (EYELA).

Polyclonal antibody production. O157, O26 and O111 extracts were prepared from bacterial strains according to the following procedure. Confluent bacterial growing on a single agar plate were harvested, washed twice with phosphate-buffered saline (PBS), and then killed in 10 % formalin-PBS for 16 h at 25℃ before being washed three times with PBS as the antigen. VT 1 and VT 2 were purified according to the following procedure. Briefly, washed *E.*

coli cells were lysed by sonication in 10 mM Tris hydrochloride (pH7.4), and the resulting lysate was precipitated with ammonium sulfate. The precipitation was dialyzed with PBS and then applied to an anti-VT antibody-bound affinity chromatography column. Purified VTs were dialyzed with PBS and concentrated before storage at 4℃.

Rabbits were immunized subcutaneously with each VT antigen in Freund's complete adjuvant four times at 2-week intervals. Small volume blood samples were taken from the rabbits to confirm antibody reactivity against the *E. coli* or VT used as the antigen. Then, all sera were collected 1 week after the last injection. Rabbit IgG was purified from sera with a Protein G column and used as polyclonal antibodies (Poly Ab).

Development of immunochromatographic tests. Test strips were prepared according to the protocol described previously (Kawatsu et al., 2006). Briefly, a 1.0-ul aliquot of Poly Ab solution (1 mg/ml) was deposited on a membrane as a 1-mm-in-width line at approximately the midpoint of the length of the membrane to serve as the test line for the *E. coli* antigen, 2 mm upstream from the midpoint to serve as the test line for VT1, and 2 mm downstream from the midpoint to serve as the test line of VT2. As the control line, 1.0 ul of a goat anti-rabbit immunoglobulin G solution (0.5 mg/ml) (Immunoprobe Co., Ltd, Saitama, Japan) was deposited on the membrane as a 1-mm-in-width line 8 mm downstream from the *E. coli* test line or the VT2 test line (Figure 1). To prepare Poly Ab-colloidal-gold conjugates, 1.0 ml of a Poly Ab solution (20 ug/ml) was added to 5 ml of a colloidal-gold suspension (40 nm; Tanaka Kikinzoku Industrial Co., Ltd) After incubation and blocking, the Poly Ab-gold conjugates were suspended in 5.0 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 1% bovine serum albumin and 1% trehalose, and were then finally absorbed with a conjugate pad and freeze-dried at -80℃. To prepare the test strips, an absorbent pad (Millipore, Bedford, MA) and the conjugate pad were attached to a laminated membrane card (HiFlow Plus 135; Millipore) in such a manner that the membrane (1 mm) and the absorbent pad (1mm) overlapped slightly.

Immunochromatographic testing of cell strains of *E. coli* and non-*E. coli* species. Cultured bacterial strains were examined using the test strips according to the following procedure. Bacterial strains incubated in untreated medium were used as samples for immunochromatography to detect E. coli O157, O26 and O111; bacterial strains incubated in polymyxin B-treated medium were used as samples for immunochromatography to detect VT 1/2. To disposable culture tubes (Fisher), 100 ul samples were added and the test strips were then inserted into the samples. After migration of the sample through the membrane for 15 min at room temperature, the appearance of red lines at both the test and the control line was interpreted as positivity for the *E. coli* antigen or the VT antigen; any other findings were interpreted as negative (positive, +; weakly positive, w+; negative, -).

Bacterial inoculation test of food samples. Food samples were purchased at a local supermarket and stored at 4℃ prior to inoculation. One ml of the 10-fold serial dilutions of each strain was added to separate 25 g food samples containing 225 ml of NmEC and homogenized in a STOMACHER400 CIRCULATOR before incubation for 22 h at 42℃. Non-inoculated food samples were also included as a control. Immunochromato testing for the detection of VT1/2 was conducted on the enrichment broth immediately after adding polymyxin B.

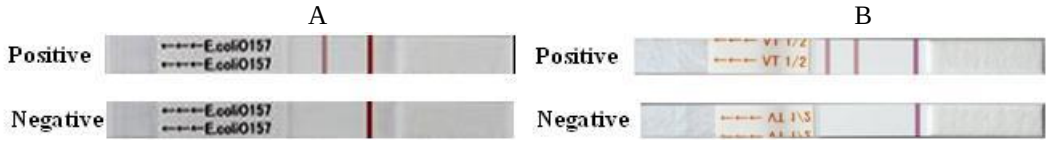

Figure Detection results with NH Immunochromato tests. (A) NH Immunochromato O157. (B) NH Immunochromato VT 1/2

III. RESULTS AND DISCUSSION

Immunochromatographic testing of cell strains of *E. coli* and non-*E. coli* species. It is necessary to examine the reactivities of test strips with various *E. coli* strains and other bacterial species before using them as a method to detect *E. coli* in food. For this purpose, cell suspensions of these bacteria were immediately treated with polymyxin B for emission of VT, and the resulting cell extracts were tested using the test strips. The results are summarized in Tables 1 and 2. All targets tested yielded positive results with the test strips. The lowest detection limit for *E. coli* was 10^4 CFU/ml of cell suspension, and those for VT1 and VT2 were 2.5 and 1.25 ng/ml, respectively. All of the non-target bacteria tested yielded negative NH Immunochromato results. Taken together, these results suggest that NH Immunochromato have sufficient sensitivity and specificity for *E. coli* detection.

Table 1 Sensitivities of NH Immunochromato tests. (A) Sensitivities of NH Immunochromato O157, O26 and O111. (B) Sensitivities of NH Immunochromato VT1/2

A

kit	strains	bacteria cells (CFU/ml)				
		1×10^7	1×10^6	1×10^5	1×10^4	1×10^3
NH Immunochromato O157	EHEC O157 ATCC35150	+	+	+	+	-
NH Immunochromato O26	EHEC O26 RIMD05091876	+	+	+	+	-
	EHEC O26 kept at Nippon Meat Packers Inc	+	+	+	w+	-
NH Immunochromato O111	EHEC O111 RIMD05091876	+	+	+	w+	-
	EHEC O111 kept at Nippon Meat Packers Inc	+	+	+	+	-

B

kit	VT	VT concentration (ng/ml)				
		10	5	2.5	1.25	0.625
NH Immunochromato VT1/2	1	+	+	w+	-	-
	2	+	+	+	w+	-

Table 2 Specificities of NH Immunochromato (A) Specificities of NH Immunochromato O157, O26 and O111. (B) Specificities of NH Immunochromato VT1/2

A

species	strains	NH Immunochromato		
		O157	O26	O111
Escherichia coli O157	ATCC25922, ATCC11775, ATCC700728, RIMD05091061	+	-	-
Escherichia coli O26	RIMD05091876、IID3005	-	+	-
Escherichia coli O111	RIMD0509829、RIMD05091865	-	-	+
Escherichia coli O1	ATCC11775	-	-	-
Escherichia coli O25	RIMD0509301	-	-	-
Citrobacter freundii	ATCC8090	-	-	-
Enterobacter aerogenes	ATCC13048	-	-	-
Enterobacter cloacae	ATCC13047、 ATCC49141	-	-	-
Enterobacter sakazakii	ATCC51329	-	-	-
Escherichia hermannii	JCM1473	-	-	-
Klebsiella pneumoniae	ATCC4352	-	-	-
Klebsiella oxytoca	ATCC8724	-	-	-
Pseudomonas aeruginosa	ATCC9027	-	-	-
Proteus vulgaris	ATCC6380	-	-	-
Serratia liquefaciens	ATCC27592	-	-	-
Serratia marcescens	ATCC8100	-	-	-
Serratia odorifera	ATCC33077	-	-	-

B

species	strains	NH Immunochromato VT1/2	
		VT1	VT2
Escherichia coli	ATCC43888, ATCC700728, ATCC25922, ATCC11775	-	-
Escherichia hermannii	RIMD05091876, IID3005	-	-
Escherichia coli O111	RIMD0509829, RIMD05091865	-	-
Citrobacter freundii	ATCC8090	-	-
Enterobacter aerogenes	ATCC13048	-	-
Enterobacter cloacae	ATCC13047, ATCC49141	-	-
Enterobacter sakazakii	ATCC51329	-	-
Klebsiella oxytoca	ATCC8724	-	-
Pseudomonas aeruginosa	ATCC9027	-	-
Proteus vulgaris	ATCC6380	-	-
Serratia liquefaciens	ATCC27592	-	-
Serratia marcescens	ATCC8100	-	-
Serratia odorifera	ATCC33077	-	-

Bacterial inoculation test in food samples. The test strips were assesed using a panel of microorganisms (Table 3). This method was used in NmEC to detect *E. coli* O157, O26, O111 and VT1/2 in food samples, after 22h enrichment. The detection limit for the assay was determined to be 0.77 CFU for *E. coli* O26, 1.5 CFU for *E. coli* O111 and, 3.3 CFU for VT 1/2 in 25g food samples (O157, data not shown) (Table3). The non-inoculated food samples were

determined to be negative for *E. coli* O157, O26, O111 and VT 1/2 by this method. In addition, this procedure can be completed with one-step incubation after the test strip has been inserted into the sample, whereas the culture method requires multiple cultures, a well-equipped laboratory and 4 to 5 days. We consider the rapidity and simplicity of the NH Immunochromato kit to make it highly suitable for use in routine screening in the food industry. The present results suggest NH Immunochromato testing, which is simple and rapid, to be useful for detecting EHEC in food samples.

Table 3 Results of the detection limit dilution assays for *E. coli* O157, O26, O111 and VT1/2 in food samples using NH Immunochromato

NH Immunochromato	**strains**	**bacterial inoculate (CFU/25g)**	**Results**	
O26	***E.coli* O26 RIMD05091876**	**0**	**-**	
		0.77	**+**	
		7.7	**+**	
		77	**+**	
O111	***E.coli* O111 RIMD0509829**	**0**	**-**	
		0.15	**-**	
		1.5	**+**	
		15	**+**	
			VT1	**VT2**
VT1/2	***E.coli* O157 kept at Nippon Meat Packers Inc.**	**0**	**-**	**-**
		0.033	**-**	**-**
		0.33	**-**	**-**
		3.3	**+**	**+**

IV. CONCLUSION

The NH Immunochromato tests (O157, O26, O111 and VT1/2) developed in this study were sensitive and specific for their targets. In addition, with these kits detection of the target antigen in a food sample was achieved within 15 min. Therefore, NH Immunochromato tests may be useful as a simple and rapid method of detecting EHEC in food.

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