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A Kit for the Detection of *Escherichia coli* Verotoxins 1 and 2
by Reversed Passive Latex Agglutination

VTEC-RPLA "SEIKEN"

For 20 tests

Among the five groups of diarrheal *Escherichia coli* (EPEC: enteropathogenic *E. coli*, EIEC: enteroinvasive *E. coli*, ETEC: enterotoxigenic *E. coli*, EHEC: enterohemorrhagic *E. coli*, and EAEC enteroadherent *E. coli*), one group is known to produce toxins which are highly cytotoxic to vero cells. These verotoxins, as they are now called, are produced by certain serotypes of *E. coli* which are associated with bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. Such verotoxin-producing *E. coli* (VTEC) are classified as enterohemorrhagic *E. coli* (EHEC) and the two serologically distinct verotoxins they produce are called verotoxin type 1 (VT1) and verotoxin type 2 (VT2).

This kit contains latex particles sensitized with highly purified antibodies specific to VT1 or VT2 and will, when mixed with culture supernatants of test isolates of *E. coli* in microplate wells, agglutinate in the presence of the respective toxin to determine verotoxin production in the strain.

CHARACTERISTICS

1. Easier to perform compared to the vero cell assay.
2. Discriminates between verotoxin type 1 and type 2.
3. Correlates well with the vero cell assay.
4. Test results are easily interpreted visually.

CONTENTS

1. Sensitized Latex VT1 5 mL x 1 vial

A suspension of latex particles sensitized with VT1 specific rabbit polyclonal antibodies containing 0.1 w/v % sodium azide as a preservative.

2. Sensitized Latex VT2 5 mL x 1 vial

A suspension of latex particles sensitized with VT2 specific rabbit polyclonal antibodies containing 0.1 w/v % sodium azide as a preservative.

3. Control Latex 5 mL x 1 vial

A suspension of latex particles sensitized with normal rabbit IgG containing 0.1 w/v % sodium azide as a preservative.

4. Control Verotoxin type 1 0.5 mL equiv. x 1 vial

Lyophilized verotoxin type 1 containing lactose and bovine serum albumin as stabilizers. Agglutination titer is shown on the vial.

5. Control Verotoxin type 2 0.5 mL equiv. x 1 vial

Lyophilized verotoxin type 2 containing lactose and bovine serum albumin as stabilizers. Agglutination titer is shown on the vial.

6. Diluent 50 mL x 1 vial

Phosphate-buffered saline (PBS) containing 0.5 w/v % bovine serum albumin and 0.1 w/v % sodium azide as a preservative.

INTENDED USE

To detect and identify verotoxins 1 and 2 in isolates of *E. coli*.

PRINCIPLE OF MEASUREMENT

Latex particles sensitized with antibodies raised against verotoxin 1 or verotoxin 2 will react to agglutinate and settle diffusely over the base of a microtiter plate well when mixed with specimens containing the respective VT; in the absence of VTs, latex particles will settle to form a tight button, a pattern easily distinguishable from positive specimens.

METHOD OF USE

Organisms identified as *E. coli* by biochemical characterization should be used as samples for this kit. Cultures derived from single, isolated colonies should be used for the assay as the presence of more than one strain may result in erroneous test results.
Although both shaking culture and stationary culture can be used when preparing specimens, stationary culture is generally recommended as it may be less influenced by culture conditions.

CONFIRMATION OF REAGENT QUALITY

Sensitized Latex VT1 and VT2 should be confirmed by showing proper agglutination with the corresponding Control Verotoxins before performing the assay.

1. Materials and reagents necessary for the test

VTEC-RPLA "SEIKEN"
A pipette (1mL), a 96well microtiter plate (V-type), a 25 μL diluter (or micropipette), a 25 μL dropper (or micropipette), a mixer for microtiter plate, black paper, plate cover, marking tape, a moisture box and a centrifuge (over 900 g or over 3000 rpm).

For stationary culture

BHI agar medium*1	Slant medium in a 30 mL test tube with cap, or agar plate in a petri dish with a diameter of 9 cm (approx. 20 mL of medium).
Polymyxin B solution	1 mL of physiological saline containing 5000 units/mL of polymyxin B sulfate dispensed into a small, centrifugable test tube.

37 °C incubator, bacteriological loop.

For shaking culture

CAYE medium *2	5 mL of medium in a 30 mL test tube with aerobic cap.

Shaker (37 °C, 120 ~150 rpm)

*1 Composition of BHI (Brain Heart Infusion) agar medium

Bovine brain, infusion from	200 g
Bovine heart, infusion from	250 g
Peptone	10 g
Glucose	2 g
NaCl	5 g
$Na_2HPO_4 \cdot 12H_2O$	2.5 g
Agar	15 g
Distilled water	1000 mL

pH 7.4

*2 Composition of CAYE (Casamino Acid Yeast Extract) medium

Casamino acid	20 g
Yeast extract	6 g
NaCl	2.5 g
K_2HPO_4	8.71 g
Salt solution *	1 mL
Distilled water	1000 mL
pH 8.5	

* Salt solution

$MgSO_4$	5 w/v %
$MnCl_2$	0.5 w/v %
$FeCl_3$	0.5 w/v %

Dissolve into 0.5 mmol/L sulfuric acid

2. Reagent preparation

Sensitized Latex VT1 and VT2	Ready to use.
Control Latex	Ready to use.
Diluent	Ready to use.
Control Verotoxin type 1 and type 2	Reconstitute each vial with 0.5 mL of diluent. Store at 2 ~ 10 °C and use within 3 months after reconstitution. If longer storage is desired, divide into small portions and store at - 20 °C or below. Do not allow repeated freezing and thawing.

3. Sample preparation

Organisms identified as *E. coli* by the biochemical characterization should be used as samples for this kit. Cultures derived from single, isolated colonies should be used for the assay as the presence of more than one strain may result in erroneous results.

4. Procedures

Although both the shaking culture and stationary culture can be used when preparing specimens. the stationary culture is generally recommended as it may be less influenced by culture conditions.

1) Cultivation of organisms

A. Stationary culture

① Inoculate the test isolate onto BHI agar medium and culture for 16 ~20 hours at 37 °C.

② Using a bacteriological loop, suspend a volume of cells equivalent to the size of 3 match heads, or all or 1/ 3 parts of the colonies on a slant culture in 1 mL of Polymyxin B solution.

③ Incubate at 37 °C for 30 minutes with intermittent shaking (every 5 ~ 10 minutes).

④ Centrifuge at 900 g for 15 minutes and use the supernatant as a test specimen.

B. Shaking culture

① Inoculate the test isolate into 5 mL of CAYE broth and incubate aerobically with shaking (120 ~ 150 strokes / min.) for 16 ~ 20 hours at 37 °C.

② Visually confirm that proper growth has occurred and then centrifuge at 900 g for 15 minutes and use the supernatant as a test specimen.

2) Procedures for Reversed Passive Latex Agglutination (RPLA) Test

Please refer to Table 1 and Figure 1 below for the procedures for reversed passive latex agglutination test.

Table 1 Dilution method

Well No.	1	2	3	4	5	6	7		8
Dilution factor	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128		Diluent control
Diluent (μL)	25	25	25	25	25	25	25	Discard	25
Sample (μL)	25	25	25	25	25	25	25	25	-
Sensitized Latex or Control Latex (μL)	25	25	25	25	25	25	25		25

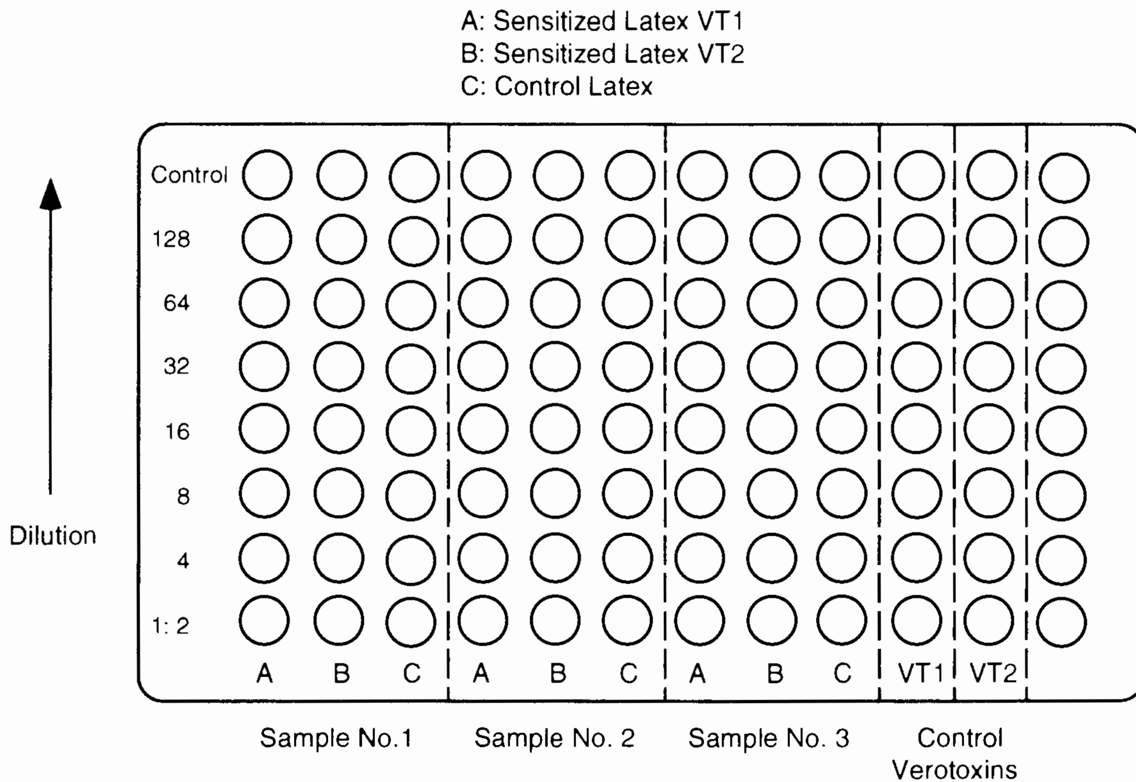


Fig. 1 Microtiter plate configuration

① Arrange and label the microtiter plate as shown in Fig. 1.

② Using a micropipette or dropper, add 25 μL of Diluent into each well of the three rows for each test specimen.

③ Using a micropipette or dropper, add 25 μL of Diluent into each well of the two rows for the Control Verotoxins.

④ Add 25 μL of the test specimen into the first wells of each of the three rows and using a micropipette or diluter, transfer 25 μL from the first well to the second and continue making two-fold serial dilutions through the 7th well, leaving the 8th well to serve as a diluent control.

⑤ In a similar manner, dilute the Control Verotoxins in the allotted wells.

⑥ Add 25 μL of Sensitized Latex VT1 into each well of the first row. 25 μL of Sensitized Latex VT2 into each well of the second row and 25 μL of the Control Latex into each well of the third row for each sample.

⑦ Similarly, add 25 μL of Sensitized Latex VT1 to each well of the row for Control Verotoxin type 1 and 25 μL of Sensitized Latex VT2 to each well of the row for Control Verotoxin type 2.

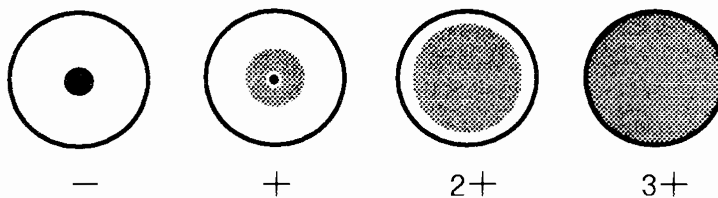
⑧ Mix the well contents by shaking the plate with the plate mixer.

⑨ After covering the plate with a lid or placing in a moisture box to avoid evaporation of the well contents, incubate in a vibration free area at room temperature for at least 16 hours and then observe the agglutination patterns.

⑩ When reading the agglutination patterns, it is helpful to place the plate on a sheet of black paper in a flat, well lit area, observing from above.

INTERPRETATION

Refer to the following figures when interpreting the results. Agglutination patterns of (+), (2+) and (3+) are considered as a positive agglutination.



Confirm that all wells for the control of diluent (the 8th wells) show a (-) agglutination pattern.

<table>
<tr><td rowspan="2">No agglutination is seen in Control Latex</td><td>Agglutination is seen with Sensitized Latex</td><td>The highest dilution factor at which agglutination is seen is used to designate the agglutination titer.
If the agglutination titer is 1 : 4 or higher, the test sample is interpreted as positive.
If the agglutination titer is 1 : 2, the sample is interpreted as indeterminate.</td></tr>
<tr><td>No agglutination is seen with Sensitized Latex</td><td>Agglutination titer of the sample is determined as less than 1 : 2 and the sample is interpreted as negative.</td></tr>
<tr><td>Agglutination is seen in Control Latex</td><td colspan="2">Interpret as indeterminate.
If the agglutination seen with Sensitized Latex is at a dilution which is four times higher (2 wells) than that of Control Latex, the test sample is interpreted as positive and the agglutination titer is determined as the highest dilution factor at which agglutination is seen.</td></tr>
</table>

NOTES ON INTERPRETATION

1) If agglutination is obtained in the diluent control well (the 8th wells), the results should be considered invalid as agglutination in this well indicates spontaneous agglutination of the latex reagent.
2) Samples which show agglutination titers greater than 1 : 128 should be retested after making a 100-fold dilution with Diluent. Calculate the agglutination titer by multiplying the newly obtained one by 100.
3) If a sample contains an extremely high titer of verotoxins, agglutination may not be observed in the wells of lower dilution due to a prozone phenomenon. In such rare cases, however, the proper agglutination will be seen in the wells of higher dilution and thus the sample can be distinguished from negative ones.
4) If no agglutination is seen with the Control Latex and the agglutination titer with the Sensitized Latex is 1 : 2 (indeterminate), the isolate should be recultured and proper growth in the culture media be confirmed. If results are still indeterminate, other test methods (e.g., vero cell assay, EIA, PCR, etc.) should be tried.
5) If a sample gives indeterminate results due to the agglutination with the Control Latex, other test methods (e.g., vero cell assay, EIA, PCR, etc.) should be tried.

PERFORMANCES

1. Sensitivity

When 25 μL of 100 ng/mL solutions of purified verotoxin type 1 or type 2 in phosphate-buffered saline containing 0.5 w/v% bovine serum albumin were used for the RPLA test, the corresponding Sensitized Latex reagents showed agglutination titers between 1 : 64 and 1 : 128.

2. Specificity

When the following strains were cultured in CAYE medium at 37 °C for 16 ~ 20 hours and then used in the RPLA assay, only strains previously confirmed to produce verotoxins were detected as positive while other strains showed negative test results.

[Strains for specificity assay]

Verotoxin producing strains

Strain	Verotoxin(s)	Strain	Verotoxin(s)
DK-EC-PS 1	VT1	DK-EC-PS 5	VT1, VT2
DK-EC-PS 2	VT1	DK-EC-PS 6	VT1, VT2
DK-EC-PS 3	VT2	DK-EC-PS 7	VT1, VT2
DK-EC-PS 4	VT2	DK-EC-PS 8	VT1, VT2

Non-producing strains

Strain	Strain
DK-EC-NS 1	DK-EC-NS 7
DK-EC-NS 2	DK-EC-NS 8
DK-EC-NS 3	DK-EC-NS 9
DK-EC-NS 4	DK-EC-NS 10
DK-EC-NS 5	DK-EC-NS 11
DK-EC-NS 6	DK-EC-NS 12

3. With-run reproducibility

When 25 μL of 100 ng/mL solutions of purified verotoxin type 1 or type 2 in phosphate-buffered saline containing 0.5 w/v% bovine serum albumin were tested 5 times simultaneously. the corresponding Sensitized Latex showed agglutination titers between 1 : 64 and 1 : 128.

4. Result of clinical trials

Correlation data with PCR and the vero cell assay in a clinical trial using 178 strains isolated from patients are shown below (performed by Tokyo Metropolitan Research Laboratory of Public Health).

Detection of VT1

		VT1 gene detection by PCR		
		Positive	Negative	Total
This kit (VT1) *1	Positive	118	0	118
	Negative	0	60	60
	Total	118	60	178

Specificity: 100 %
Sensitivity: 100 %

Detection of VT2

		VT2 gene detection by PCR		
		Positive	Negative	Total
This kit (VT2) *1	Positive	121	0	121
	Negative	0	57	57
	Total	121	57	178

Specificity: 100 %
Sensitivity: 100 %

		Vero cell assay		
		Positive	Negative	Total
This kit *1	Positive	147	0	147
	Negative	0	31	31
	Total	147	31	178

Specificity: 100 %
Sensitivity: 100 %

The above numbers represent the number of strains giving the results indicated.
*1 : The same results were obtained between stationary culture and shaking culture.

Details of strains tested
VT1 producing strains: 26, VT2 producing strains: 29,
VT1 and VT2 producing strains: 92 and non-VT producing strains: 31.

NOTES ON MEASUREMENT

1. Only colonies derived from a single, isolated strain should be used in the assay as the presence of more than one strain may result in erroneous results.
2. Preparation of samples should be performed using the culture media recommended and conditions given in this kit insert. In shaking culture method, the amount of verotoxin produced may be reduced if shaking is insufficient. After culture, proper cell growth should be confirmed.
3. Complete pelleting of cells should be confirmed after the centrifugation steps when preparing specimens from shaking culture procedure or after the Polymyxin B extraction step in the stationary culture procedure. If pelleting is not complete, centrifugation should be repeated.
4. Samples should be prepared without delay after their cultivation. If it is necessary to store samples for later testing, they should be stored at - 20 °C or lower.

PRECAUTIONS

1. General precautions

1) This kit should be used for in-vitro diagnostic purposes only.
2) This kit is intended for the detection and identification of verotoxins from isolates of *E. coli.* As many different strains of *E. coli* may exist in patient stools and, furthermore, VTEC may or may not predominate in patient stool, it is important to test as many isolates as possible for verotoxin detection. Negative test result obtained from a single isolate does not preclude the possibility that the patient is in fact not infected with VTEC.
3) Since there are reports that *Enterobactor cloacae, Citrobactor freundii* and other bacteria can produce verotoxins, it is necessary to use isolates confirmed to be *E. coli* in order to detect VTEC.
4) All patient specimens as well as the Control Verotoxins, equipment used in the test should be treated as potential biohazards and handled with appropriate caution.
5) Variant verotoxins which differ in amino acid sequence from VT1 or VT2 are known to exist and the reactivity between these variants and the Sensitized latex in this kit have not been fully characterized. External studies have shown, however, that Sensitized Latex VT2 does react with VT2c producers, for example, although possibly with reduced sensitivity.

2. Precautions for handling

1) Do not freeze the Sensitized Latex and Diluent. Freezing and thawing the reagents may give erroneous test results. The kit should be brought to room temperature (15 ~ 25 ℃) approximately 30 minutes before use.
2) Reagent vials should be shaken before use by gently inverting several times.
3) Microplates without any obvious scratches or stains should be used for the assay.
4) Reagents with different lot numbers should not be interchanged or mixed for use.
5) The test should be carried out according to the procedures in this kit insert.
6) Do not interchange caps between different reagents.

3. Precautions for waste

1) The reagents in this kit (Sensitized Latex, Control Latex, Diluent and reconstituted Control Verotoxin) contain 0.1 w/v % sodium azide as a preservative. As sodium azide may react with lead or copper piping to form explosive metal azides, they should be disposed of by flushing with copious amounts of water.
2) All specimens and equipment used in the test should be sterilized by one of the following methods after use:
   1) Soaking in 2 w/v % glutaraldehyde for 1 hour or longer.
   2) Soaking in 0.1 w/v % sodium hypochlorite for 1 hour or longer.
   3) Autoclaving at 121 ℃ for 20 minutes or longer.

STORAGE AND SHELF LIFE

Storage : 2 - 10 ℃ protected from light.
Shelf life: Up to the expiry date on the label.

PACKAGE

VTEC-RPLA "SEIKEN" for 20 tests x 1

REFERENCES

1) Riley, L. W., et al.: Hemorrhagic colitis associated with a rare *Escherichia coli* serotype., N. Engl. J. Med., 308, 681 (1983).
2) Karmali, M. A. : Sporadic cases of haemolytic uremic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. Lancet, 1. 619 (1983).
3) Karmali, M. A. : Infection by Verotoxin-producing *Escherichia coli*., Clin. Microbiol. Rev., 2, 15(1989).
4) Konowalchuk, J., et al. : Vero response to a cytotoxin of *Escherichia coli*., Infec. Immun., 18, 775 (1977).
5) Adrienne, W. P., et al. : *Enterobactor cloacae* producing a Shiga-like toxin II - related cytotoxin associated with a case of hemolytic-uremic syndrome, J. Clin. Microbiol., 34, 464 (1996).
6) Herbert, S., et al. : Shiga-like toxin II - related cytotoxin in *Citrobactor freundii* strains from human and beef samples, Infec. Immun., 61, 534, (1993).
7) Marques LRM., et al. : *Escherichia coli* strains isolated from pigs with edema diseases produce a variant of shiga-like toxin II., FEMS Microbiol. Lett., 44, 33 (1987).