Effects of Disinfectants on Shiga-Like Toxin Converting Phage from Enterohemorrhagic *Escherichia coli* O157 : H7

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(Received January 9, 2001; Accepted January 29, 2001)

Inactivation of free phage carrying *slt*2 from enterohemorrhagic *Escherichia coli* (*E. coli*) O157 : H7 by four kinds of common disinfectants in Japan was examined under conditions with (dirty) and without (clean) interfering substance. Ethanol (EtOH) inactivated the phage within one minute under both conditions. The effect of sodium hypochlorite (NaOCl) on this phage decreased under the dirty condition, but was potentiated by increasing the concentration and contact time to the degree that could be sufficient for practical use. Use of benzalkonium chloride (BAC) at a high concentration: 0.2%, would be effective. Alkyldiaminoethylglycine hydrochloride (DAG) was not effective on this phage.

Key words —— *Escherichia coli* O157 : H7, *slt*2-converting phage, disinfectant, inactivation

INTRODUCTION

Epidemics of food/water-borne infection of enterohemorrhagic *Escherichia coli* (*E. coli*) O157 : H7 (O157 : H7) have occurred worldwide including outbreak caused by hamburger ingestion in Oregon and Minnesota states in USA,[1] and have been attracting attention as an emerging infectious disease. In Japan, outbreak of O157 : H7 infection occurred in a kindergarten in U City, Saitama Prefecture, in which two children died, and outbreak occurred in various regions including Sakai City, Osaka prefecture, in 1996, which generated more than 9000 patients. Thereafter, many incidences of O157 : H7 infection including sporadic cases have occurred, becoming a serious social issue.

It was initially revealed by Scotland *et al*.[2] in 1983 that the gene (*slt*) encoding Shiga-like toxin (SLT = Verotoxin : VT) in enterohemorrhagic *E. coli* is carried by lysogenic phage similar to λ phage in *E. coli* O26 : H11. In 1984, O'Brien *et al*.[3] showed that production of SLT in O157 : H7 is also due to lysogenic phage. O'Brien *et al*. further reported that this phage is induced by UV irradiation and transduced *E. coli* K12 strain. Muehldorfer *et al*.[4] lysogenized *E. coli* C600 with *slt*1-carrying phage (H19B) or *slt*2-carrying phage (933W) and induced the phage by treating the bacteria with mitomycin C. Tanaka *et al*.[5] reported that *slt*-carrying phage was induced not only by UV irradiation and treatment with mitomycin C but also by mutagens 3-amino-1-methyl-5H-pyrido (4,3-b) indole (Trp-P-2) and 2-amino-3-methylimidazo (4,5-f) quinoline (IQ) and DNA synthesis inhibitor ofloxacin, and the induced phage caused phage conversion in JM109 derived from *E. coli* K12. Induction of SLT production by 4-quinolone was reported by Kimmitt *et al*.,[6] and induction of phage carrying *slt* gene by norfloxacin was reported by Matsushiro *et al*.[7]

In 1998, Muniesa and Jofre[8] demonstrated the presence of free phage carrying *slt* in sewage. This phage caused phage conversion in *E. coli* ATCC 43888 (O157 : H7; *slt*$^-$) and produced SLT.

The presence of free infectious lysogenic phage in the environment and induction of *slt*-carrying phage by mutagenic treatment and 4-quinolones suggest possible horizontal transmission of *slt* via phage, and the potential risks cannot be excluded. Therefore, in this study, we investigated the effects of common disinfectants in Japan on free phage carrying *slt*.

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MATERIALS AND METHODS

Preparation of *slt*2 Carrying Phage Suspension and Counting of Phage —— For the bacteria donating *slt*2 carrying phage, an SLT-producing strain (9705 strain) isolated from feces from an asymptomatic individual was selected from O157 : H7 isolates obtained at Tokyo metropolitan Arakawa Health Center. The phage is spontaneously induced in 9705 strain.[5] Strain 9705 was cultured in brain heart infusion (BHI) broth after incubation with shaking at 37°C overnight. The culture fluid was centrifuged in a microtube (Ultrafree MC) attached with a membrane filter (0.45 μm) at 15000 rpm for 30 min, and the filtrate was used as a phage suspension. The presence of *slt* in the phage suspension was confirmed in polymerase chain reaction (PCR)-amplified product. To 1.2 ml of the phage suspension, 250 μl of 20% polyethyleneglycol (PEG 6000)-2.5 M NaCl was added to concentrate, and the phage was treated with 100 μl of Tris-$MgCl_2$ and 2 μl of D Nase I (2.5 mg/ml)/R Nase A (1.0 mg/ml) solution at 37°C for one hour. The suspension was re-treated with 20% PEG 6000-2.5 M NaCl, then treated with 25 μl of 4% SDS and 20 μl of proteinase (1 mg/ml) in 175 μl of TE buffer at 37°C for one hour. The DNA was extracted with phenol/chloroform and precipitated by ethanol. The DNA precipitated was resuspended in sterile distilled water, and amplified according to the instruction attached to Takara one shot screening kit (Takara Shuzo, Japan). The amplified product was confirmed as a band corresponding to 171 bp on a 4% Nusieve® 3 : 1 agarose (BioWhittaker Molecular Application) by agarose gel electrophoresis.

The presence of phage was confirmed by plaque formation on an agar plate in which JM109 strain derived from *E. coli* K12 was mixed. JM109 strain was cultured in BHI broth with shaking at 37°C for 18 hr. The culture fluid was seeded in BHI broth at a ratio of 1 : 1000, and cultured with shaking at 37°C until OD_{660} reached 0.4, then 100 μl of this culture fluid,10 μl of phage solution, and 3 ml of λ soft agar were mixed. The mixture was overlaid on a BHI agar plate and incubated at 37°C overnight. Formation of turbid plaques was confirmed the number of phages in the test phage suspension was measured as described above. The transduced stain picked from the plaque was confirmed to carry *slt* using the PCR method described above.

Disinfectants —— Disinfectants examined by the phage inactivation test were 80% (v/v) ethanol (EtOH), sodium hypochlorite (NaOCl), benzalkonium chloride (BAC), and alkyldiaminoethylglycine hydrochloride (DAG). The concentrations examined are conventional concentrations of these disinfectants.

Phage Inactivation Test —— To 24-well microplates, 800 μl of each disinfectant diluted with sterile distilled water was added to each well. In the test under the clean condition, 100 μl of sterile distilled water was added. In the test under the dirty condition, 100 μl of dry yeast suspension adjusted to a final concentration of 2% was added. For the control, 800 μl of sterile distilled water was added in place of the disinfectant. To each test disinfectant solution, 100 μl of the phage suspension was added and incubated at room temperature. After 1, 5, 10, 30, and 60 min, 100 μl of the reaction mixture was added to 1.9 ml of neutralizing agent (peptone : 1 g, Tween 80 : 5 g, sodium thiosulfate : 1 g, lecithin : 0.7 g/l) to inactivate the disinfectant. On a agar plate, 200 μl of the neutralized solution, 100 μl of incubated JM109, and 3 ml of λ soft agar were mixed and overlaid, then the plate was incubated at 37°C overnight. Plaques formed on the plate were counted and the inactivation effect of each disinfectant was evaluated. It was confirmed that the neutralizing agent does not affect infectivity of the phage and growth of the indicator bacteria before the test.

RESULTS AND DISCUSSION

Regarding the effects of disinfectants on phage carrying *slt* from O157 : H7, Karch *et al*.[9] investigated the inactivation effect of four disinfectants containing glutaral or BAC on phage 933J reported by O'Brien *et al*.[3] and SLT-producing phage derived from a clinical isolate. However, there has not been any report of common one-component disinfectants in Japan. Therefore, we prepared phage suspension using 9705 stain that spontaneously induces phage carrying *slt*2, and the inactivation effects of four disinfectants: EtOH, NaOCl, BAC, and DAG at the conventional concentration, were examined under clean condition and dirty condition with 2% dry yeast imitating a fecal condition. The numbers of phages after treatment with disinfectants are graphed in Figs. 1–5.

On the test of EtOH, no plaques were formed by phage treated for one min under the clean condition

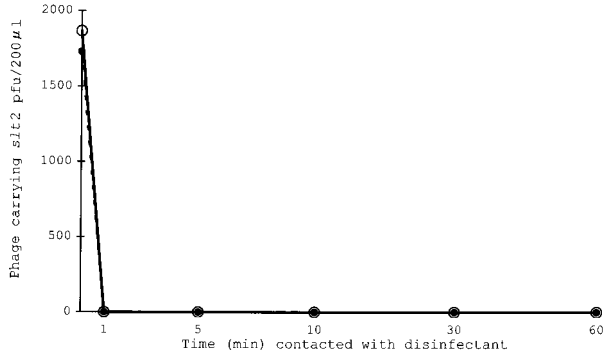

Fig. 1. Inactivation of Free Phage Carrying *slt*2 by 80% (v/v) EtOH
—○— clean condition
·····●····· dirty condition
Experimental conditions are shown in the part of MATERIALS AND METHODS.

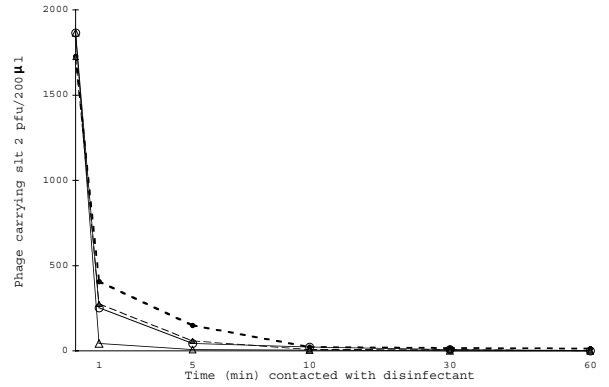

Fig. 2. Inactivation of Free Phage Carrying *slt*2 by 0.02% and 0.05% NaOCl
—○— 0.02% clean condition —△— 0.05% clean condition
·····●····· 0.02% dirty condition —▲— 0.05% dirty condition
Experimental conditions are shown in the part of MATERIALS AND METHODS.

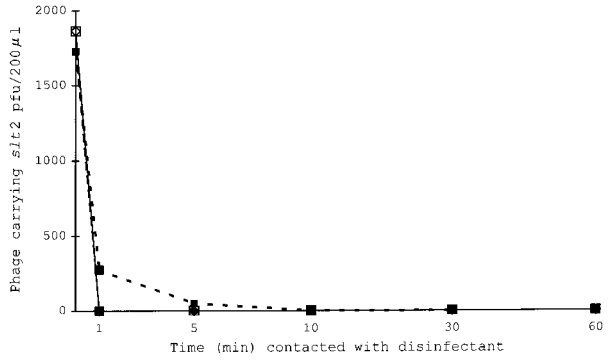

Fig. 3. Inactivation of Free Phage Carrying *slt*2 by 0.1% and 1% NaOCl
—□— 0.1% clean condition —◇— 1% clean condition
·····■····· 0.1% dirty condition —◆— 1% dirty condition
Experimental conditions are shown in the part of MATERIALS AND METHODS.

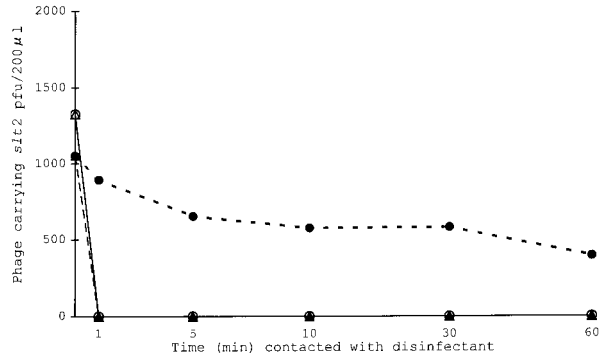

Fig. 4. Inactivation of Free Phage Carrying *slt*2 by 0.05% and 0.2% BAC
—○— 0.05% clean condition —△— 0.2% clean condition
·····●····· 0.05% dirty condition —▲— 0.2% dirty condition
Experimental conditions are shown in the part of MATERIALS AND METHODS.

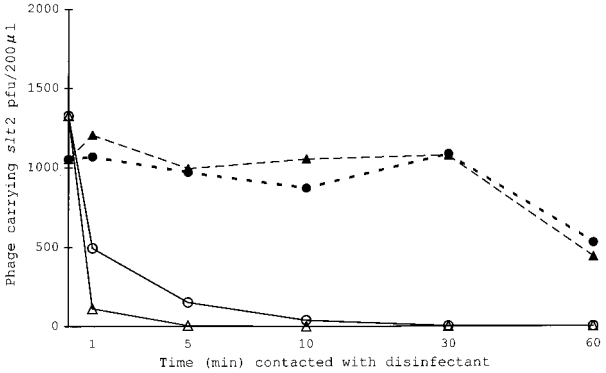

Fig. 5. Inactivation of Free Phage Carrying *slt*2 by 0.05% and 0.2% DAG
—○— 0.05% clean condition —△— 0.2% clean condition
·····●····· 0.05% dirty condition —▲— 0.2% dirty condition
Experimental conditions are shown in the part of MATERIALS AND METHODS.

and even in the presence of 2% dry yeast (Fig. 1). Therefore, EtOH is useful as an immediately acting disinfectant in the presence and absence of organic substances.

On the test of NaOCl under the clean condition, no plaques were formed by phages treated with 0.02% solution for 60 min, 0.05% solution for 30 min, or 0.1% solution for one min. Under the dirty condition, plaques were observed after treatment with 0.02% and 0.05% solutions for 60 min, but no plaques were formed after treatment with 0.1% solution for 30 min or 1.0% solution for one min. NaOCl is generally likely to be affected by organic substances. However, in the presence of 2% dry yeast although the effect decreased after treatment at low concentrations for short time the inactivation after

10 min or longer treatment with NaOCl was comparable to that under the clean condition. Muniesa *et al.*[10] reported that *slt*-carrying phage in swage was more resistant to chlorine and heat than O157 : H7. As shown in Figs. 2 and 3, the inactivation effect of NaOCl on the phage increased as the concentration increased and the contact time increased in both clean and dirty conditions. Therefore, NaOCl is a useful disinfectant against to the phage carrying *slt*.

On the test of BAC, no plaques were detected after treatment with 0.05% solution for one min under the clean condition. Under the dirty condition, approximately 40% of the phages survived after treatment with 0.05% solution for 60 min, but no plaques were detected after treatment with 0.2% solution for one min (Fig. 4). Low-level disinfectants such as BAC and DAG are regarded to be ineffective on non-enveloped viruses and likely to be affected by organic substances.[11] However, on this study, the phage was inactivated within one min by treatment with 0.05% BAC solution under the clean condition. Under the dirty condition, BAC was not affected by 2% dry yeast solution, and the phage was inactivated. Therefore, BAC at a relatively high concentration may be effective in practical use.

On the test of DAG under the clean condition, phages were inactivated by treatment with 0.05% solution for 60 min and 0.2% solution for 10 min. Under the dirty condition, approximately 40% of the phages survived after treatment with 0.2% solution for 60 min (Fig. 5). DAG is generally regarded to be unlikely to be affected by organic substances.[12] However, in this study, the inactivation effect of DAG was the lowest among the disinfectants tested, and the inactivation effect in the presence of 2% dry yeast was unexpectedly low.

As described above, O'Brien *et al.*[3] reported in 1984 that the gene encoding SLT, the major pathogenic factor of O157 : H7,is carried by lysogenic phage. Tanaka *et al.*[5] in our group reported that mutagenic substances such as Trp-P-2 and IQ and ofloxacin induced *slt*-carrying phage in O157 : H7, and the phage infected JM109 strain derived from *E. coli* K12 and changed the phenotype. Kimmitt *et al.*[6] reported that ofloxacin, ciprofloxacin, and nalidixic acid induced production of SLT2, and Matsushiro *et al.*[7] reported that norfloxacin induced *slt*-carrying phage. Muehldorfer *et al.*[4] reported that RecA is involved in induction of phage by mitomycin C, and Kimmitt *et al.*[13] noted that induction of *slt*-carrying phage by 4-quinolones is due to SOS response. Acheson *et al.*[14] demonstrated that *slt*-carrying phage (H19B) infects other serotype of *E. coli* (AK16) and lysogenizes in mouse intestine. These findings indicate that integrated *slt*-carrying phage is easily induced, and the induced phage infects other strains of *E. coli*.

The presence of free *slt*-carrying phage in sewage has been reported by Muniesa and Jofre[8] in 1998. Later, they surveyed 33 rivers in 10 countries in Europe and other region, and detected *slt*2-carrying phage in 15 points.[15] They noted that *slt*-carrying phage survives longer than O157 in sewage, and *slt*2 is more stable in free phage than in the host genome, and thus, transmission of *slt*2 via phage in the environment may be an important infectious factor.[8,10]

Kogure and Ikemoto[16] showed that O157 : H7 could enter the viable but nonculturable (VNC) state in sterilized natural river water. Approximately 100 CFU/ml bacteria in natural river water from five points in city areas reacted with anti-O157 antibody. They considered that O157 : H7 is very likely to exist in VNC state in natural river water.

Tani and Nasu[17] detected O157 : H7 in VNC state in a river water environment using *in situ* PCR and fluorescent antibody methods. They hypothesized the presence of infectious phage, and suggested the horizontal transmission of *slt* and the presence of *E. coli* with different antigenicity other than O157 : H7 and other bacteria, emphasizing the necessity of tracing *slt*.

O157 : H7 exists in rivers, and *slt*-carrying phage integrated in the host is easily induced by DNA damaging substances and infects other *E. coli*. Moreover, free *slt* carrying phage constantly exits. These facts indicate potential risk of the bacteria carrying this phage, which should be a concern for public health. Therefore, it is important for hygiene to report information of effects of common disinfectants on free *slt*-carrying phage and to make the information common knowledge.

Acknowledgements We deeply appreciate Tokyo metropolitan Arakawa Health Center for providing *E. coli* O157 : H7 9705 strain.

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