

SIMULTANEOUS EFFECTS OF SHAKING AND TEMPERATURE ON VEROTOXIN1 PHAGE INDUCTION FROM VEROTOXIGENIC ESCHERICHIA COLI STRAINS

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ABSTRACT

Induction of lambda phage carrying verotoxin1 gene from a verotoxigenic strains of *Escherichia coli* and released verotoxin1 were studied under environmental factors of shaking and temperature. Verotoxin1 phage in *Escherichia coli* PA 101 and transductants was confirmed by bacteriophage detection assay. Shaking of culture media and increasing temperature until 42 °C increased phage particles in supernatants of *Escherichia coli* PA 101. Our results indicate that environmental factors such as shaking movements in natural habitats of bacteria such as river or sewage streams and temperature rise in summer season could be factors in induce and release free verotoxin1 – producing phage particles in nature that in turn could be the source of phage spreading to other related bacteria, and responsible for increased outbreaks of food borne diseases with verotoxigenic *Escherichia coli* in warm months of year in tropical areas.

Key words: Verotoxigenic *Escherichia Coli*, lambda bacteriophages, verotoxin1

INTRODUCTION

Verotoxin producing *Escherichia coli* (VTEC) are responsible for sporadic cases of diarrhea and have occurring world wide by ingestion of hamburgers. (Paton *et al.*, 1998) VTEC causes severe clinical manifestations such as haemorrhagic colitis and haemolytic uremic syndromes. These strains produce shiga like toxins. Verotoxin genes in VTEC are encoded by lambdaoid bacteriophage family, that could be induced by factors such as irradiation and mitomycin C. Subinhibitory concentrations of selective antimicrobials for treatment of VTEC outbreaks (Quinolones and macrolides) also could induce verotoxin genes in VTEC strains. Induction of this genes in turn could be increase verotoxin1 titer in culture or natural environments of bacteria (Yoh *et al.*, 1999). Muniesa and Jofre reported that free phages carrying verotoxin genes are present in sewage. This phages lysogenized *E.coli* ATCC (43888, O157: H7, st1) and produced Verotoxin (Muniesa *et al.*, 1998). Muniesa reported that VTEC bacteriophages are more resistant to chlorination

and pasteurization, survive better in water than their bacterial hosts. Therefore free living phage particles are potential factors in the spreading and transmission of verotoxin genes among diverse groups of *E.coli* and related bacteria (Muniesa *et al.*, 1999). Presence of free infectious phage particles in the environment and potential risk of horizontal transmission to other related bacteria, alerts health care authorities and professionals about risks of VTEC caused disease (Schmidt *et al.*, 1999). These facts indicate that in natural habitats some factors may be help to the induction and spreading of phage particles to other bacterial hosts. In this study we investigate the effects of two environmental factors shaking and temperature on the induction of verotoxin1 bacteriophage from a VTEC.

MATERIALS AND METHODS

Bacterial strains and cell lines: PA101 is a non-O157 VTEC isolated from a non symptomatic healthy person that produces only verotoxin1, provided by Dr.M. M. Aslani, Pasteur institute of Iran. *E.coli* DH5 α was used for transduction experiments. Vero lines was purchased from cell

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bank of Pasteur Institute of Iran.

Growth media and verotoxin1 measurement
PA101 strains was grown in Trypticase Soy Broth. Overnight cultures were washed several times with fresh media. Verotoxin1 Phage was confirmed by plaque forming assay in PA 101 strain. Verotoxin1 concentration in broth or bacterial pellets were determined using a reverse passive latex agglutination (VTEC-RPLA, E.coli verotoxin detection kit; Denka Seiken. Co,Tokyo, Japan) which can differentiate verotoxin1 and verotoxin2 with anti-Vt1 antibody sensitized latex and anti-Vt2 antibody sensitized latex, in accordance with the manufacturers procedure. Peroplasmic toxin extracted by polymyxin B sulphate (0.3 mg/mL final concentration). Cytotoxic activity of crude and affinity purified verotoxin1 tested in a vero cell assay system.

Effects of shaking and temperature on induction of bacteriophage and verotoxin1 titer

E.coli PA101 was cultured overnight at 37 °C on tow sets of TSB without shaking ; and TSB at 150 rpm. Verotoxin1 titer and plaque forming units of phage particles in supernatants determined by VTEC-RPLA and bacteriophage detection methods, respectively. In other experiment, we cultured E.coli PA101 overnight at 37 °C in TSB with 150 rpm. Then broth divided in 15 sections in tubes with 5 mililiter and incubated in 37 °C , 38 °C, and two fold increase from 40 to 62 °C for 10 hours. Then verotoxin1 titer and phage particles were determined as previously mentioned.

Determination of plaque forming units

Plaque forming unit experiments was done by E.coli DH5α as a recipient host. E.coli DH5α and PA101 were cultured in luria broth and in TSB (150 rpm) at 37 °C overnight respectively. Liquid culture of PA101 was centrifuged (5000g) at 4°C, for 10 min. Supernatant sterilized through 0.22 μm pore size filters. Dilutions of filterate was mixed with 0.5 Mcfarland concentratation of E.coli DH5α, and presence of phage was detected by plaque assay on LB soft agar with %0.7 agarose(Maniatis et al., 1982).

Verotoxin1 production in transductants of DH5α (TDH5α) was confirmed by VTEC-RPLA Kit in comparison with negative controls of DH5α.

RESULTS

Kinetic of verotoxin1 production in culture media

Time dependent evaluation of verotoxin1 production indicate that in solid TSB,verotoxin1 detected in pellets of bacteria 3 h after bigining of culture. But in TSB without shaking and TSB with 150 rpm detectable verotoxin1 titer measured after 12 and 4 hours of cultures respectively. PA101 growth in TSB (150 rpm) resulted in a 4 fold increase in yield in comparison with TSB without shaking. Shaking of PA101 strongly increased verotoxin1 amount and phage particles in supernatant. We found less than 5 percent of verotoxin1 in supernatant of unshaked TSB cultures of PA101. Similarly plaque assay of filter sterilized supernatants of unshaked TSB showed low levels of phage particles (Fig. 1).

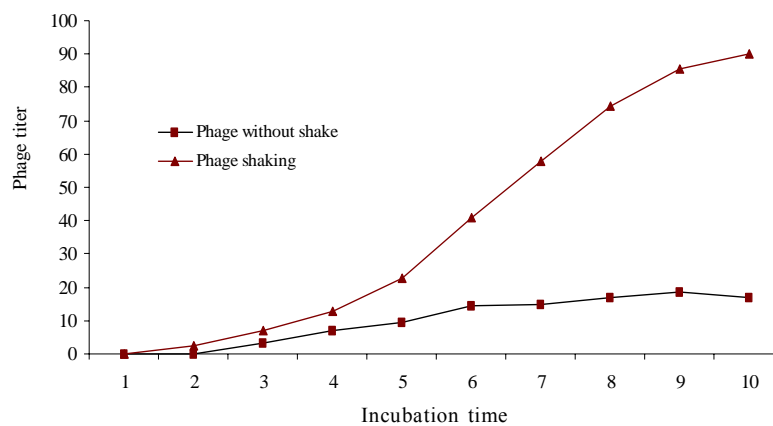


Fig. 1: Phage particles in plaque assay method in broth cultures in shaking and without shaking cultures

Study of temperature effects on verotoxin1 production indicate that in TSB cultures of PA101 at 37 °C to 55 °C, verotoxin1 titer increased with elevation of temperature, but decreased in higher than 55 °C. Low levels of verotoxin1 presence in

62 °C incubated samples of PA101 culture (Fig. 2) indicates that pasteurized methods of foods such as milk for inactivation of toxins must be reevaluated.

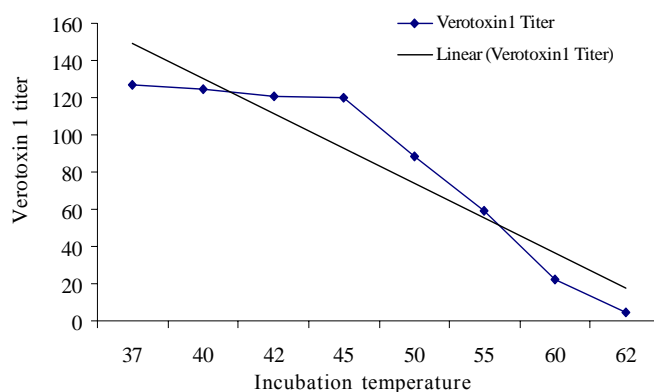


Fig. 2: Verotoxin titer in different incubation temperatures

Lysigenization of *E.coli* DH5 α

Filter sterilized supernatant of *E.coli* PA101 grown on TSB (150 rpm) was used to transduce *E.coli* DH5 α . Transduced bacteria (TDH α) produced high titer of verotoxin1 in TSB at 150rpm shaking. verotoxin1 phages in TDH α was confirmed by plaque assay on Luria broth low and top agars. Production of verotoxin1 in TDH α strain evaluated by VTEC-RPLA Kit. Lysates and supernatants of both PA101 and TDH α had same specificity for killing cultured vero cells. Intraperitoneal injection of both supernatants in Balb/c induced paralysis of hind-leg and death in second week of injection.

DISCUSSION

Virulence genes are frequently encoded in mobile genetic elements, including bacteriophages. This genes are aquired by pathogenic bacteria via lateral transfer of these elements. (Acheson *et al.*,1998) Our studies indicate that verotoxin1 bacteriophage could be easily transfer horizontally to other related bacteria, and environmental factors such as shaking of contaminated material and temperature fluctuations could also help to releasing and inducing verotoxin1 phage in natural habitats. Kohler reported that lambdoid phages of *E.coli* in vivo induced under poor environmental conditions such as starvation,aggressive chemicals,

and UV light. (Kohler *et al.*,2000) Muniesa reported that verotoxin1 producing bacteriophages survive better in water than their bacterial hosts and are more resistant to chlorination and pasteurization. (Muniesa *et al.*, 1998) Kohler reported that subinhibitory concentrations of the antibacterials growth promoters of quinoxaline-1,4 -dioxide- type antibiotics olquinox and carbodox increased the release of STX-converting phage particles from Shiga toxin producing *E.coli* (Kohler *et al.*, 2000). Kimitt reported that quinolones such as ciprofloxacin, ofloxacin, and nalidixic acid have inducing potentials for verotoxin2 production. (Kimmitt *et al.*, 2000)Acheson reported that SLT-carring phage (H19B) have potential of infecting other serotypes of *E.coli* (Acheson *et al.*, 1998). Feng reported that coliphages MS2 and Q β have different inactivation rates in a range of pH and temperature(Feng *et al.*, 1992) Helge indicated that clinical SLT-producing *E.coli* isolates lost their toxin genes during subcultivation and become nontoxigenic(Karch *et al.*, 1992) Our results in lossing of verotoxin1 gene in broth cultures are in accordance with Helge studies. Kajiura assesed the potential of four free phages carrying slt genes from Enterohemorrhagic *E.coli* O157:H7 (Kajiura *et al.*, 2001) Ohnishi reported that horizontal gene transfer plays central role in generating genomic diversity among *E.coli* strains(Ohnishi *et al.*, 2001).

Our results indicate that verotoxin1 phage and toxin amount in in vitro increased after shaking and incubation of grown strains in higher temperatures. These findings show that antibacterials and environmental conditions contribute to induction and releasing of verotoxin1 phage in different biological ecosystems, and could help to spreading of verotoxin-producing genes among bacterial hosts and producing new Verotoxin producing E.coli strains. In addition, presence of verotoxin1 in high-temperature incubated broths of E.coli PA101 indicate that fast pasteurization methods for foods such as milk could have risks for public health and necessitates exact quality control for pasteurization processes.

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