CHARACTERIZATION OF RECOMBINANT ANTIBODIES DEVELOPED FOR CAPTURING ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

Reka Kanitpun[1], G Gale Wagner and Suryakant D Waghela

Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas, USA

Abstract. *Escherichia coli* O157:H7, an emerging cause of food-borne disease with the occurrence of an estimated 20,000 illnesses and 250 deaths each year in the United States, has now been reported from several countries worldwide. Infections with this bacteria, which follows the ingestion of contaminated food by humans, causes bloody diarrhea, hemolytic uremic syndrome (HUS), and renal disease, that can have serious health implications. The source of food contamination is usually associated with animals, mainly cattle. Many cattle become infected early in life when they are exposed to an environment that is contaminated by other animals shedding the organisms in their feces. Detection of *E. coli* O157:H7 in feces or contaminated food samples requires tests with high sensitivity, which is increased by the use of monoclonal antibodies. However, the production of concentrated monoclonal antibodies in ascites raises animal welfare concerns, and can be expensive. In this study, single chain of variable fragment (scFv) molecules were developed from hybridoma clones that produce immunoglobulins specific for the LPS and flagella antigen of *E. coli* O157:H7 using phage display technology. The reactivity of the soluble scFv for their respective antigens was preserved in ELISA and by partial inhibition of bacterial agglutination with polyclonal antiserum. Furthermore, the scFv were able to capture *E. coli* O157:H7 bacteria demonstrating their potential use in diagnostic assays.

INTRODUCTION

Escherichia coli O157:H7, a serotype in the enterohemorrhagic group of *E. coli* (EHEC), was first identified in 1982 by the Center for Disease Control and Prevention (CDC) following two bloody diarrhea outbreaks in humans linked to the same fast food restaurant chain (Griffin and Tauxe, 1991). *E. coli* O157:H7, which causes an estimated 60 deaths and 73,000 illnesses annually in the US (Riley, 1987) has now been recognized as an important cause of bloody diarrhea in more than 30 countries on six continents (Griffin *et al*, 2002). Most *E. coli* O157:H7 infections are mild and do not require medical care. However, some result in hemorrhagic colitis, which typically begins with a sudden onset of severe abdominal cramps and a watery diarrhea that may become grossly bloody. Most patients recover in 6 to 8 days after onset since the illness is usually self-limiting. However, some people, particularly the very young and immunosuppressed individuals, may develop hemolytic-uremic syndrome (Riley *et al*, 1983). The EHEC strains produce phage encoded verocytotoxins and are able to produce attaching-effacing (A/E) lesions in the animal host. There are more than 200 serotypes of *E. coli* that express the verocytoxins also known as Shiga-like toxins (O' Brien and La Veck, 1983; Nataro and Kaper, 1998). Thus, the identification of *E. coli* O157:H7 can be problematic since failure to identify this organism may lead to delayed recognition of outbreaks as well as disease control measures. Culture methods in different selective media have been developed, but seem to be inadequate in confirming the presence of *E. coli* O157:H7 (Fukushima *et al*, 2002). PCR assays have also been developed, including multiplex PCR (Paton and Paton, 2002), with a variety of primers to amplify verotoxin genes. However,

Correspondence: SD Waghela, Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467, USA.
Tel: 979-845-5176; Fax: 979-862-1147
E-mail: swaghela@cvm.tamu.edu

[1]Present address: National Institute of Health, Kasetklang, Chatuchak, Bangkok 10800, Thailand.

there are still disadvantages to this method, such as inhibitors in samples that affect the sensitivity (Thomas *et al*, 1994), and the occurrence of false positive results. Finally, the extremely small number of the bacteria found in contaminated samples may be undetectable even by PCR. Monoclonal antibodies (mAbs) (Kohler and Milstein, 1975) and polyclonal antibodies have been utilized in several immunoassays to detect *E. coli* O157:H7 (Kim and Doyle, 1992; Pyle *et al*, 1999; Shelton *et al*, 2003). Modifications of the immunoassays use the antibodies to capture the bacteria from various types of samples to increase the sensitivity in subsequent procedures (Wright *et al*, 1994; Chapman and Siddons, 1996; Chapman *et al*, 1997). MAbs are very important research tools, and have been used in the development of several diagnostic tests (Mori *et al*, 1987; Feld *et al*, 1992). Although antibody-producing hybridoma clones may be grown in mass culture, the procedure can be expensive. Alternatively, highly concentrated mAb requires ascites production in mice. This procedure has animal welfare implications and several European countries have legislation limiting antibody production in mice (Marx *et al*, 1997).

Recombinant antibody engineering based on the phage display system (Barbas, 1993; Burton, 1995) in which fragments of functional antibodies are displayed by expression as fusion proteins on the tips of the filamentous phage surface, has revolutionized the field of epitope specific antibody production and application (McCafferty *et al*, 1990; Burioni *et al*, 1998). The smallest functional fragments of antibodies that maintain the binding and specificity of the whole antibody is made up of only the variable regions of light and heavy chains (V_L and V_H). These fragments are called Fv (variable region fragment). Stable Fvs can be produced by making recombinant molecules in which the V_L and V_H domains are connected by a flexible peptide linker of 15-20 amino acids as a single molecule, the single-chain Fv fragment (scFv) (Winter *et al*, 1994).

For this study, murine hybridomas producing mAbs that react with the O157 lipopolysacchride and H7 flagella were used to make the cDNA for the amplification of V_H and V_L genes (Huse *et al*, 1989; Horton, 1995). These two genes linked as scFv genes were cloned into phagemid for expression and selection for reactivity to either antigen. The scFv proteins specific for the LPS and H7 of *E. coli* O157:H7 were characterized and used to capture *E. coli* O157:H7.

MATERIALS AND METHODS

Preparation of antigens

Preparation of LPS antigen. The procedure of LPS extraction from *E. coli* O157:H7, a Shiga-toxin negative strain (# 43888, ATCC, Rockville, MD, USA) was a modification of the aqueous phenol extraction method (Westphal and Jann, 1965). Briefly, bacterial cells from overnight culture growth in LB broth medium were harvested, washed once with distilled water, the pellet resuspended in 2.5 x (v/w) of deionized water and the suspension heated at 68ºC for 15 minutes. An equal volume of 90% (w/w) phenol also heated at 68ºC was added to the bacterial suspension, stirred at 68ºC for 15-20 minutes, then chilled on ice and centrifuged at 3,000*g* for 30 minutes for separation of the phases. The phenol plus interphase layers were re-extracted with water. The combined upper aqueous phases were dialyzed against deionized water and the dialysate incubated at 50ºC for 1 hour following the addition of Proteinase K (50 μg/ml). The LPS in the dialysate was precipitated by adding 2.5 x volumes of acetone and incubating at 4ºC overnight. The LPS precipitate was pelleted by centrifugation at 10,000*g* at 4ºC for 30 minutes.

Preparation of H7 flagella. *E. coli* O157:H7 was grown overnight under static, non-aerated conditions at 37ºC in Penassay broth (Difco Labs, Detroit, MI, USA). The bacteria were harvested by centrifugation at 5,000*g* for 30 minutes and the pellet containing the bacteria was resuspended in sterile 0.9% normal saline. To dissociate the flagella from the bacteria, the pH of the bacterial suspension was adjusted to 2.0 with 1 N HCl and maintained at that pH with constant stirring for 30 minutes. The bacterial cells, which were now devoid of flagella, were removed by centrifugation at 5,000*g* for 30 minutes. The supernatant containing detached flagellin was further centrifuged at 100,000*g*

for 1 hour at 4ºC to sediment the insoluble material, and the pH of the supernatant adjusted to 7.2 with 1 N NaOH. The flagella protein was precipitated with the addition of ammonium sulfate (final concentration 2.67 M) and stirred for 16 hours at 4ºC. The precipitate, collected by centrifugation at 15,000*g* for 15 minutes at 4ºC, was dissolved in distilled water and dialyzed against running tap water initially for 2 hours and then against several changes of deionized water for 18 hours at 4ºC with constant stirring (Sherman *et al*, 1988).

Hybridoma cell lines

Hybridoma cell lines CRL-2507 ($IgG_{3,}$ Kappa) and CRL-2509 (IgG_1, Kappa), which produce mAbs that react with the lipopolysaccharide, O157, and the flagella antigen, H7, respectively of *E. coli* O157:H7, were purchased from ATCC, Rockville, MD, USA. The hybridoma cells were grown in RPMI 1640 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine solution, 50 mg/ml gentamicin at 37ºC in a humid atmosphere containing 5% CO_2 (Pearson *et al*, 1980). The secreted mAb in the supernatant was tested for reactivity to the antigen in an ELISA and its concentration estimated using the quantitative ELISA kit for mouse IgG (Bethyl Labs, Montgomery, TX, USA).

cDNA synthesis

RNA was extracted from $5x10^7$ hybridoma cells producing the relevant mAbs using a single step isolation method (Chomczynski and Sacchi, 1987). The mRNA was purified from total RNA using Oligotex® mRNA spin columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cDNA was reverse transcribed by heating the mRNA to 65ºC for 10 minutes and incubating for 60 minutes at 37ºC with a mixture of Moloney murine leukemia virus (M-MLV), reverse transcriptase, BSA, deoxynucleotides, DTT solution, and random primer $pd(N)_6$ mix.

Construction, amplification, and ligation of scFv

The primary PCR reactions were performed in a 50 µl final reaction volume using the immunoglobulin gene primers (V_HBack (5′) AGGT (GC) (AC) A(AG)CTGC AG(GC) AGTC(AT)GG (3′), V_HForward (5′) TGAGGAGACGGTGACCGT GGTCCCTTGGCCCC (3′), V_KBack (5′) GACA TTGAGCTCACCCAGTCTCCA (3′), V_KForward (5′) CCGTTTTATTTCCAGCTTGG TCCC (3′). Each reaction contained 25 pmols of either V_HBack and V_HForward primer mix for heavy chain amplification, or V_KBack and V_KForward primer mix for light chain amplification, and 10 mM dNTP, 5 µl of cDNA template, 1x PCR buffer and 1 unit of $Vent_R$DNA polymerase (New England Biolabs, Inc, Beverly, MA, USA). The amplification was carried out using an initial denaturation step at 95ºC for 7 minutes, followed by 30 cycles of amplification with denaturation at 94ºC for 1 minute, annealing at 55ºC for 2 minutes, and extension at 72ºC for 2 minutes. The heavy and light domains were assembled into a single gene by splicing an overlap extension (Horton, 1995) using a linker fragment (LINKforward (5′) TGGAGACTGGG TGAG CTCAATGTC (3′) and LINKback (5′) GGGACCACGGTCACC GTCTCCTCA (3′)) coding for a flexible $(Gly_4Ser)_3$ peptide. In the secondary PCR amplification, the assembled fragments were amplified by using two oligonucleotide primers that contain SfiI and NotI restriction sites to facilitate directional ligation into the phagemid vector. However, prior to ligation into the phagemid, the scFv gene of about 800 bp was inserted into the pCR 2.1-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) and transformed into chemically competent Top10F' one shot competent cells (Invitrogen, Carlsbad, CA, USA) for nucleotide sequencing. The sequencing was performed at the Gene Technology Laboratory, Department of Biology, Texas A&M University. Nucleotide sequence analysis was performed with Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). Homology searches were performed with the BLAST (http://www.nlm.nih.gov/).

Selection, expression and purification of soluble scFv

The scFv sequences were found to have significant homology to various scFv sequences from the Gene Bank database and the DNA digested with SfiI and NotI was directionally ligated into pCANATB 5E (Amersham Pharmacia, Piscataway, NJ, USA) and then used to transform into the competent cells of *E. coli* strain TG1 (Amersham

Pharmacia, Piscataway, NJ, USA). After the transformed cells were grown in media containing 100 μg/ml of ampicillin, $4x10^{10}$ pfu of M13 KO7 helper phage was added to rescue the recombinant phage particles. The recombinant phage was precipitated by PEG/NaCl and collected by centrifugation at 10,000*g* for 20 minutes at 4°C. Phage libraries were screened and enriched for antigen specific clones by solid phase panning using 25 cm^3 tissue culture flasks coated with either immobilized LPS (10 μg/ml) or the flagella (10 μg/ml) of *E. coli* O157:H7 in 5 ml coating buffer (0.06 M carbonate buffer pH 9.6). To minimize non-specific binding, the flasks were pretreated with blocking buffer (3% bovine serum albumin in PBS) for 1 hour at room temperature, and then washed 3 times with PBS. The precipitated recombinant phage diluted in blocking buffer containing 0.1% Triton X-100 was added to the antigen coated flask, and then incubated at 37°C for 2 hours. Unbound phages were removed by decanting the suspension, and washing each flask 20 times with PBS by filling and emptying the flask completely, and then 20 times with PBS containing 0.1% Tween 20. TG1 cells were added directly to the washed panning flask and incubated at 37°C for 1 hour with intermittent gentle shaking for the infection of bacteria with phage bound to flagella antigen/LPS flask surface. The infected TG1 cells were amplified, and the phage rescued for a second round of panning by repeating the infection cycle. The final bacterial suspension harvested after the second panning was plated on a selective agar medium containing nalidixic acid and ampicillin to grow the transformants. These transformants were used for further selection and characterization of the expressed scFv. An ELISA was used for screening positive clones. The wells of microtiter plates were pre-coated with either LPS or the flagella of *E. coli* O157:H7 at a concentration of 10 μg/ml coating buffer. Each step of the ELISA was followed by a wash step consisting of 3 washes with PBS-T. The blocked wells in the plates were filled with 100 μl of the supernatant harvested from each scFv clone and incubated for 1 hour at room temperature. Anti-E Tag mAb (Amersham Pharmacia, Piscataway, NJ, USA), diluted to 1 μg/ml was added to each well and the plate incubated for 1 hour at room temperature. An HRP-conjugated goat anti-mouse IgG antibody (Sigma, St Louis, MO, USA) diluted to 1:5,000 was added to each well and the plate incubated 1 hour at room temperature. The binding of the 2nd antibody to the mAb, scFv, and antigen complex was visualized by the addition of 2′, 2′ azino-bis (3-ethylbenzhiazoline-6- sulphonic acid) (ABTS; KPL, Bethesda, MD, USA) to each well and the absorbance read at 405 nm. Four clones with high absorbance values were selected for plasmid isolation to confirm the insertion of the scFv gene, and to confirm the nucleotide sequence.

Four TG1 clones producing anti-H7 scFv, that gave a higher intensity in the ELISA, were used for preparing recombinant phage to infect the *E. coli* strain HB2151 (Amersham Pharmacia, Piscataway, NJ, USA) for protein expression. The infected HB2151 cells were grown on 1.5% (w/v) agar medium made of 0.2% (w/v) Bactotryptone, 0.05% (w/v) Bacto-yeast extract, and 0.05% (w/v) NaCl, 0.01M $MgCl_2$, 0.1M glucose, 100 μg/ml of ampicillin, 0.4 mM nalidixic acid. Ten single colonies were inoculated separately in 50 ml of 2YT broth medium containing 100 μg ampicillin/ml and 2% glucose and incubated at 30ºC overnight with rotational shaking at 250 rpm. After pelleting at 1,500*g* for 10 minutes, the cells from a 5 ml aliquot were resuspended in 50 ml of 2YT broth medium containing 100 μg ampicillin/ml and 0.3 mM/l of IPTG, and further incubated for 3 hours at 30ºC with rotational shaking at 250 rpm. This induced culture was centrifuged at 1,500*g* for 20 minutes, the supernatant collected, filter sterilized for storage at 4ºC, and the pellet used for periplasmic protein extraction. The cell pellets expressing anti-H7 scFv in 1/50th the original culture volume of cold 0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, and 0.5 M sucrose (TES). The cell suspension was allowed to incubate on ice for 30 minutes following the addition of and mixing with 1.5 volumes of 0.2X TES to provide an osmotic shock. After centrifugation at 5,000*g* for 10 minutes, the supernatant containing the soluble periplasmic proteins was collected separately from the insoluble cell extract (Plückthun and Skerra, 1989).

For producing scFv for the O antigen, four TG1 clones harboring plasmids containing the scFv gene from hybridoma clone for O antigen

were used for preparing recombinant phage to infect *E. coli* strain XL1-Blue (Startagene, La Jolla, CA, USA). Ten colonies of the transformed *E. coli* XL1-Blue were separately grown, induced with 0.3 mM IPTG and were lysed in B-PER® protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). The soluble protein containing the scFv was separated from the insoluble material by centrifugation at 27,200*g* for 15 minutes.

Since the recombinant scFv carry a C-terminal 13 amino acid peptide tag (E-Tag), an anti-E Tag monoclonal antibody affinity column (HiTap column, Amersham Pharmacia, Piscataway, NJ, USA) was used for purification of the scFv preparations.

SDS-PAGE analysis and Western immunoblotting

Samples were reduced and denatured for 4 minutes at 100°C in sample buffer and the proteins separated by electrophoresis through 12.5% SDS-polyacrylamide gel (SDS-PAGE). The separated proteins were visualized by Coomassie blue staining (Barbet *et al*, 1983). Proteins in duplicate gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) (Towbin and Gordon, 1984). The molecular weights were estimated using prestained SDS-PAGE markers (BIO-RAD, Hercules, CA, USA). Western blots were processed as previously described (Waghela *et al*, 2000), except that the membranes were blocked with 2% casein in PBS for 30 minutes before adding anti-E Tag antibody (8 μg/ml in 1% casein in PBST) for 2 hours at room temperature. An alkaline phosphatase-conjugated goat anti-mouse antibody diluted 1:5,000 in PBST was used before the detection of the antibody binding by 5-bromo-4-chloro-3-indoxyl phosphate, disodium salt, and nitroblue tetrazolium substrate (Sigma Chemical St Louis, MO, USA).

Binding activity of scFv in an ELISA

To check the binding activity of scFvs, 96-well microtiter plates (Immulon®-2, Dynatech Laboratories, Chantilly, VA, USA) were coated with either the LPS or the flagella protein of *E. coli* O157:H7 at a concentration of 1 μg/100μl/well in coating buffer and incubated at 4°C overnight. The subsequent steps were as described above. The antigen in the coated wells was reacted with the scFv or mAb, prior to the addition of anti-E Tag mAb for the detection of bound scFv. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (whole molecule) (Sigma, St Louis, MO, USA) diluted 1:5,000 was then added to each well. The enzyme conjugated antibody was then detected by color development following the addition ABTS. The enzyme activity in each well was measured by absorbance at 405 nm. Controls included wells that were coated with a purified *Salmonella enteritidis* Typhimurium LPS (Sigma, St Louis, MO, USA) and H antigen from a motile non-O157:H7 *E. coli*, kindly provided by Dr T Omran (Texas A&M University).

Competitive ELISA assay

This assay was performed as above except that ~20 μg of either of anti-O157 scFv or anti-H7 scFv were separately premixed with 50 μl of polyclonal antibodies against LPS and H7 of *E. coli* O157:H7 (Lee Laboratories, Grayson, GA, USA). These scFvs mixed with the rabbit antisera, at different dilutions (1:10, 1:20, 1:40, 1:80 and 1:160), were added into each well to compete for binding and incubated for 1 hour at room temperature. The control wells used normal rabbit serum. The bound scFv was then detected by anti-E tag mAb with subsequent steps as above.

Inhibition of agglutination assay

The inhibition of agglutination of *E. coli* O157 H7 by anti-O157 and anti-H7 rabbit serum was tested with anti-O157 and anti-H7 scFv. The bacteria were grown overnight at 37ºC in Penassay broth. One hundred μl of each culture was mixed with each soluble scFv at final concentrations of 0.0 μg, 0.5 μg, 1.0 μg, 2.0 μg and 3.0 μg, then incubated for 1 hour at 37ºC. Then, each polyclonal antibody diluted to 1:50 in PBS was added (100 μl/well) and the plate incubated overnight at 37ºC. Agglutination was observed and compared to control wells containing bacteria with only polyclonal antibody.

Capture assay

Wells of a microtiter plate were coated at 4ºC overnight with 1, 2, and 4 μg of soluble scFvs (either anti-O157 or anti-H7) in 100 μl of coating buffer. The parental mAbs for these two scFvs

and a blank well (data not shown) were used as control. The plates were washed 3 times with PBS and blocked with 2% casein in PBS for 1 hour at room temperature. One hundred µl of an overnight culture of *E. coli* O157:H7 was added to each well. A similar culture of a motile strain of *E. coli* was added for control in duplicate coated wells. The plates were incubated for 2 hours at room temperature, and then washed 3 times with PBST and 3 times with PBS. One hundred µl of fresh LB medium was then added into each well and incubated at 37°C for 1 hour with gentle shaking. From each well, 100 µl of culture was plated on LB agar and incubated at 37°C overnight. Colony counts were determined for capture assay analysis. This assay was done in duplicate for calculation of the mean value.

RESULTS

The template cDNA, prepared by reverse transcription of mRNAs isolated from CRL-2507 and CRL-2509 hybridoma cell lines, gave amplified products of about 344 and 320 bp for V_H and V_L, respectively by PCR (Fig 1A). The authenticity of the amplified product was confirmed by sequencing the V_L and V_H products for submission to BLAST (data not shown). The scFv genes for each hybridoma cell line were prepared for ligation into a pCANTAB 5E phagemid by linking both V_H and V_L PCR products using a $(Gly_4Ser)_3$ linker and restriction site primers SfiI and NotI, which yielded a product of approximately 800 bp (Fig 1B). The *scFv* genes from both the cell lines were then sequenced following insertion into pCR 2.1- TOPO plasmid. The aligned sequences from 4 clones of each scFv showed good homology to other immunoglobulin gene sequences when submitted to Ig BLAST. The homology of the sequences, which ranged from 89-96%, confirmed that the sequences were from Ig genes. The scFv genes were then directionally ligated into the phagemid vector DNA for transforming *E. coli* TG1 cells following digestion of the original PCR products with restriction enzymes with SfiI and NotI. The transformed *E. coli* TG1 culture supernatants containing phage were added on to O157 and H7 antigens immobilized on the solid matrix for selecting binding clones by panning. Following a second panning of the selected phages, the supernatants of 72 clones were used to identify clones that had a higher reactivity with the relevant antigen in an ELISA. The presence of scFv insert in four clones of each scFv-prep with the highest absorbance value was confirmed by restriction digestion of the extracted plasmid DNA and nucleotide-sequencing. The amino acid sequences deduced from the nucleotide sequences showed the relevant components of the expected fusion protein (Fig 2). Phages from these four clones were transfected into either *E. coli* HB2151 *E. coli* XL-1 Blue strain for protein expression. Ten subclones for anti-H7 scFv (pRKCANTABO1 – pRKCANTABO10) were picked from transformed HB2151 strain and ten for anti-O157 scFv (pRKCANTABO1 – pRKCANTABO10) were selected from transformed XL-1 Blue strain. A representative clone, pRKCANTABO1.6 developed from the mAb hybridoma for the LPS - O157 expressed ~36-kDa scFv as a soluble protein in the XL1-Blue strain (Fig 3, lanes 1 and 2). Similarly, the scFv developed from H7 reacting hybridoma was expressed in supernatant and periplasmic spaces as a protein of ~30 kDa in HB2151 strain (Fig 3, lanes 7 and 8).

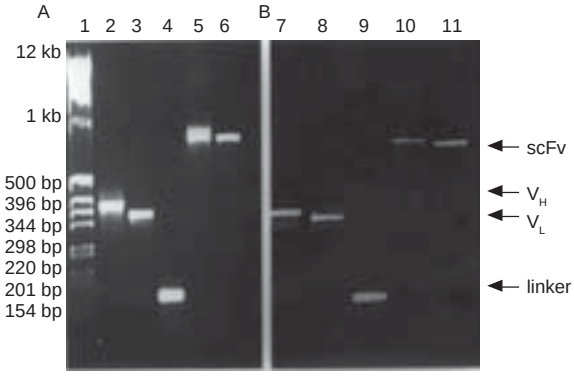
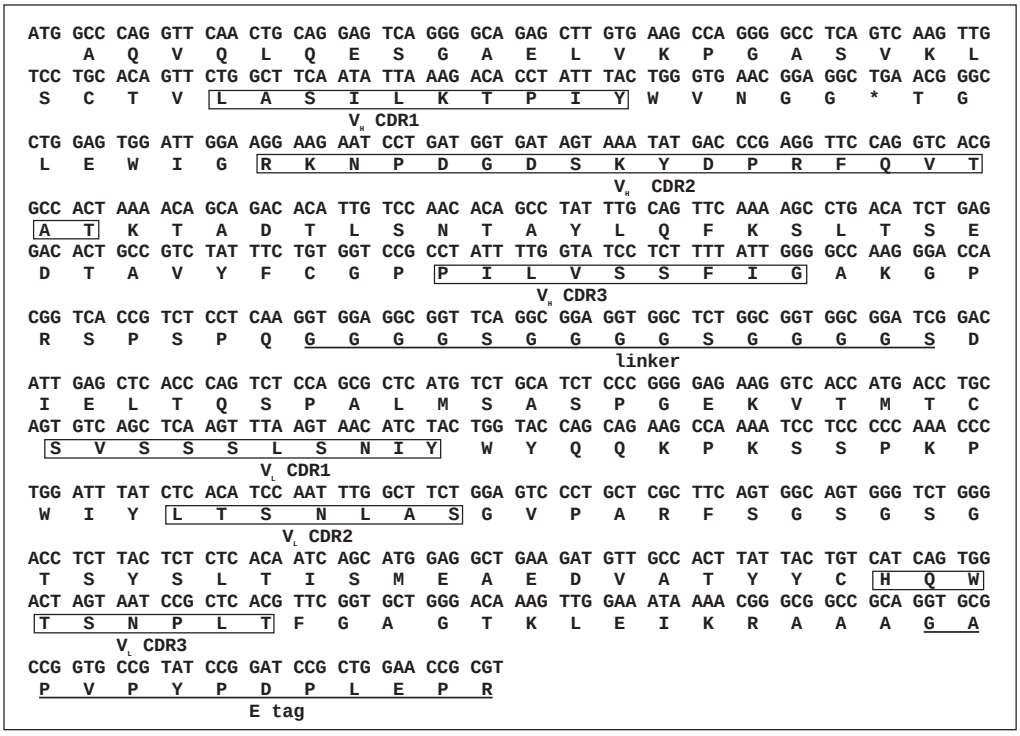


Fig 1–Amplification of V_H, V_L, linker and scFv products. For hybridoma clone producing mAb against LPS of *E. coli* O157:H7 (A) and for hybridoma clone producing mAb against H7 antigen *E. coli* O157:H7 (B). The amplicon DNA was visualized by UV light following staining of DNA separated by electrophoresis through a 1.5% agarose gel. Lanes: 1, 1Kb DNA marker (BioRad, Hercules, CA, USA); 2, 7 -V_H PCR product (~344bp); 3, 8 - V_L PCR product (~320bp); 4, 9 - linker PCR product (~93bp); 5, 10 - scFv PCR product (~800bp); 6, 11 - scFv PCR product after restriction digestion with SfiI and NotI (~800bp).



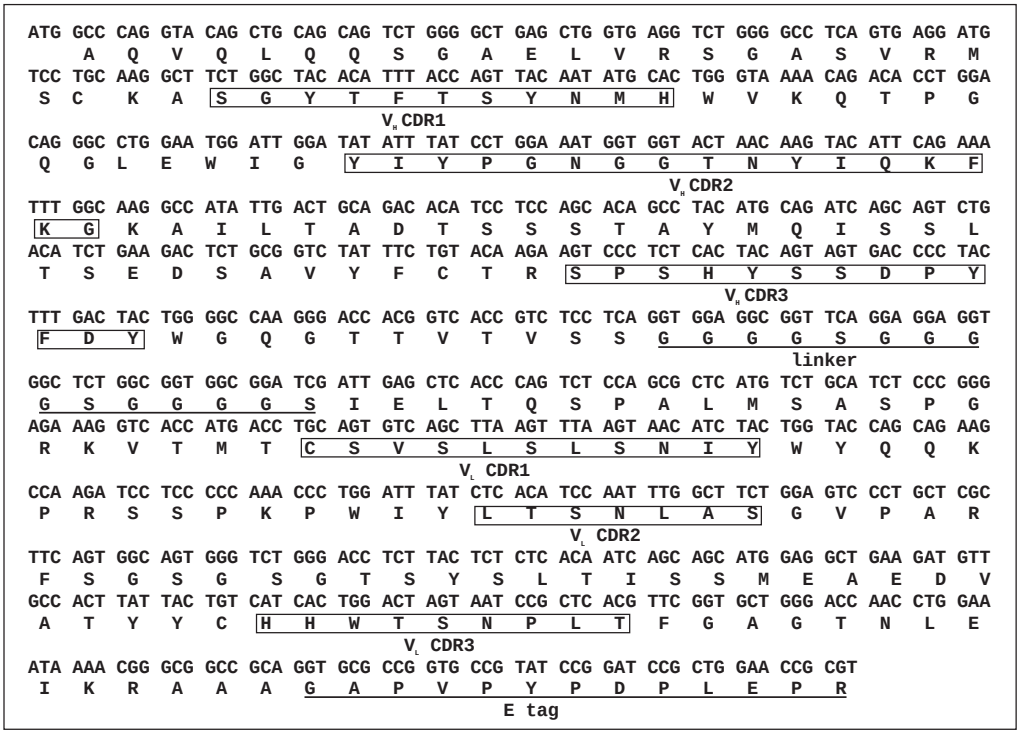


Fig 2–Nucleotide and deduced amino acid sequence of anti-O157 scFv (upper box) anti-H7 scFv (lower box). Complementary determining regions (CDRs) are boxed. The $(Gly_4Ser)_3$ linker and E-tag sequences are underlined.

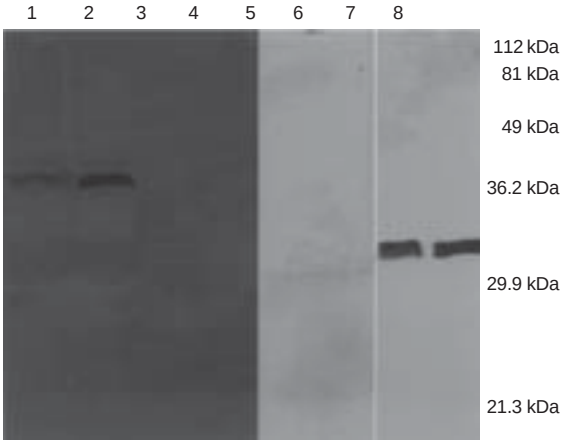


Fig 3–Western immunoblot of the expressed scFvs detected using anti E-Tag monoclonal antibody. Lanes 1, 2 - soluble protein extract from an anti-O157 scFv sub-clone; 3, 4 - supernatant from an anti-H7 scFv sublcone; 5, 6 - insoluble cell extract of the same anti-H7 scFv subclone; 7, 8 - periplasmic extracts from the same anti-H7 scFv subclone.

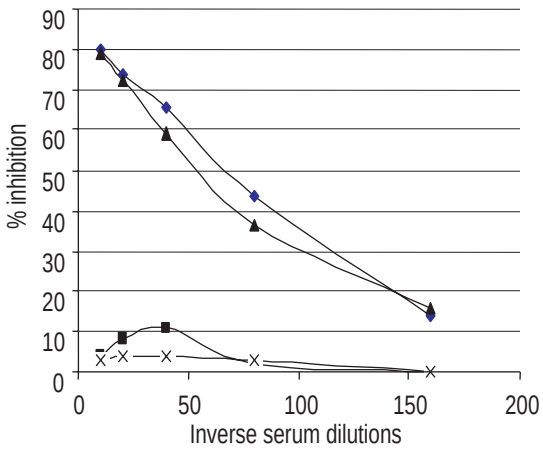


Fig 4–Inhibition by polyclonal antibody against O157 (◆) and H7 (▲) in competitive ELISA of anti-O157 scFv and anti-H7 scFv binding with O157 LPS antigen and H7 antigen, respectively. Control normal rabbit serum competing with anti-O157 scFv (■) and anti-H7 scFv (X).

The ELISA assays showed that the soluble extracts from anti-O157 scFv producing clones reacted to LPS antigen of *E. coli* O157:H7, but not to the LPS of *S. enteritidis* Typhimurium. Similarly, the soluble scFv in the supernatants and periplasmic extracts from a representative clone pRKCANTABH4.1 and 4.2 reacted to the H7 antigen but not to H antigen from the non-relevant motile strain of *E. coli*.

In competitive ELISA assay, extracted protein from inclusion bodies for O157 antibody and purified soluble scFv for H7 antibody were mixed with different dilutions of each of the polyclonal antibodies. This mixture than competed for binding to the relevant antigen as indicated by the differences in absorption at OD 405 nm. The inhibition was greater in the presence of a higher concentration of polyclonal serum. The inhibition was 14, 44, 65.3, 73.8, and 79.2 % for dilutions 1:160, 1:80, 1:40, 1:20, and 1:10, respectively, for anti O157 polyclonal antibody binding to its antigen (Fig 4, Top). Similarly, the inhibition for anti-H7 polyclonal antibody was 16, 36.7, 59, 72, and 79 % for dilutions 1:160, 1:80, 1:40, 1:20, and 1:10, respectively (Fig 4, Bottom).

Polyclonal antibody against either the O157 LPS or the H7 flagella agglutinated suspensions of *E. coli* O157:H7. The degree of agglutination (determined visually by comparison with the control) was reduced in the presence of increasing concentrations of the respective scFv (Table 1).

Compared to an irrelevant scFv (anti-F5) 4 μg of both anti-O157 scFv and anti-H7 scFv captured *E. coli* O157:H7. The ability to capture *E. coli* O157:H7 was reduced with decreasing concentrations of the scFv for anti-O157 (2,100 and 450 CFU/ml at concentrations of 2 μg and 1 μg, respectively), and for anti-H7 (870 and 510/ml CFU for concentrations of 2 μg and 1 μg, respectively). The parental mAb of each scFv used as control also captured the *E. coli* O157:H7 (Table 2). However, both the scFv and the mAbs did not capture the motile non-O157:H7 *E. coli* in the assay (data not shown).

DISCUSSION

E. coli O157:H7 will continue to be an important public health concern as long as it contaminates meat and other food products. The risk of human infection decreases with the speed of detection of a source of an outbreak, hence the method of diagnosis should be fast and accurate. Such rapid tests should be used proactively to identify the

Table 1
Agglutination inhibition assay. The reduction in the degree of agglutination of *E. coli* O157:H7 with the respective polyclonal antisera following the addition of either the anti-O157 scFv, the anti-H7 scFv or the anti-F5 scFv to the reaction.

	Anti-O157 scFv (μg)				Anti-H7 scFv (μg)				Anti-F5 scFv (μg)			
	3	2	1	0.5	3	2	1	0.5	3	2	1	0.5
Antiserum												
O157	+++*	++	+	-	-	-	-	-	-	-	-	-
H7	-	-	-	-	+++	++	+	-	-	-	-	-

*Inhibition of agglutination: 4+ = 100%; 3+ = 75%; 2+ =50%; + =25% and - = 0%

Table 2
Capture assay. The mean number of colony forming units of *E. coli* O157:H7 captured by either anti-O157 or anti-H7 scFv and their parental mAbs.

Capturing reagent	Mean cfu/ml*
Anti-O157 scFv - 2 μg	2,100
1 μg	450
Anti-H7 scFv - 2 μg	870
1 μg	510
Anti-O157 mAb - 2 μg	4,150
Anti-H7 mAb - 2 μg	1,500

* - mean of two tests.

original sources of infection in animals. When this happens, the economic impact caused by the recall of large quantities of meat, for example, will be drastically reduced. Many improved methods have been developed for diagnosis, however, they seem to have limitations, such as the use of special selective media for culture, the need for specialized equipment and skills for assays such as the PCR. The immunoassays for the detection of *E. coli* O157:H7 are mainly based on the use of monoclonal antibodies. Monoclonal antibodies are often used in diagnostic tests of various other diseases as well (Mori *et al*, 1987; Feld *et al*, 1992; Choi *et al*, 2003). However, the cost and the animal welfare issues associated with the production of monoclonal antibody are of great concern. The alternative method of genetically engineering immunoglobulin molecules may provide advantages in such tests, compared to the monoclonal antibody. Engineered antibody fragments, in the form of scFv, have been used, for example, for the detection of exposure to Foot-and-Mouth disease virus and anthrax (Zhou *et al*, 2002). ScFvs have the same specificity for an epitope as the parental monoclonal antibody (Casalvilla *et al*, 1999; Nagesha *et al*, 2001). Such molecules can be used instead of the monoclonal antibody currently employed in assays to capture *E. coli* O157:H7 for further confirmatory tests such as isolation of the bacteria or PCR. We have engineered two scFvs that react with the O157 LPS and H7 flagella and have shown that they can be used for capturing *E. coli* O157:H7 bacteria.

The first objective of this study was to construct *scFv* genes using the hybridoma cells that produce the anti-O157 LPS and the anti-H7 flagella monoclonal antibodies. These genes were then expressed in *E. coli* strains for the production of soluble scFv using phage display technology. The phagemid carrying the inserted scFv genes were used for transformation of *E. coli* TG1 cells, which allows the read through of the amber codon at the end of the E-tag. Then, phages are released with the aid of a helper phage M13KO7. The phages with the fusion of scFv and g3p exposed on the surface can be used for biopanning for selecting and enriching the clones with activity against a particular antigen. Although *E. coli* TG1 is a suppressor strain, the suppression of the amber codon is about 20 % efficient, thus soluble antibodies (scFv) produced from the *E. coli* TG1 harboring the desired phage can be used to select clones that express the recombinant protein. In the present study, one clone of each from 72 that reacted to either O157 or H7 antigen was selected to produce a larger amount of soluble antibody

using the non-suppressor HB2151 strain of *E. coli* in which the translation aborts at the end of E-tag. As expected, the anti-H7 scFv was expressed as a soluble antibody by this strain. However, anti-O157 scFv, which has extra amber codon present in its gene, was not produced. Therefore, another strain, *E. coli* XL1-Blue, a suppressor strain which allowed the scFv to be expressed as ~38-kDa fusion protein of *fd* gene 3, was employed. The fusion protein was extracted as soluble inclusion bodies, which reacted with antigen in ELISA tests. Therefore, the extra amino acids of the fusion with the *fd* gene 3 do not seem to interfere with the ability to bind antigen.

Expression was not a problem for the anti-H7 scFv since the protein was detected in the periplasmic extracts of the *E. coli* H2151 strain, and was produced as the expected ~30 kDa protein. However, the amount produced was low. For better production, the *scFv* gene needs to be transferred to a different vector. This needs to be done for anti-O157 *scFv* gene as well, but for the different reason of the presence of the *fd* gene3 fusion (ShengFeng *et al*, 2003). The reactivity of both the soluble scFvs was shown in an ELISA format. Reactivity was inhibited to various degrees in the presence of monospecific polyclonal antisera at different concentrations, indicating the specificities of the scFvs. This specificity was further confirmed by the inhibition of agglutination of *E. coli* caused by these antisera in the presence of various concentrations of the scFv. Next, the scFvs were used to capture *E. coli* O157:H7 cells. One μg of the scFv was able to capture sufficient numbers of the bacteria such that a 1-hour incubation of the captured bacteria resulted in ~500 cfu/ml. This suggests that scFvs can be modified to capture bacteria from contaminated food products or fecal material of infected animals as has been done previously with antibody coated magnetic beads (Wright *et al*, 1994; Chapman and Siddons, 1996). This would provide a cost-effective procedure since an average of 20-200 mg of scFv per liter of medium may be produced using a suitable vector-host system (Gavilondo and Larrick, 2000). Such scFv-based rapid test would allow for a wider application and be useful in the detection of infected animals for epidemiological and risk assessment studies.

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REFERENCES

Barbas CF 3rd. Recent advances in phage display. *Curr Opin Biotechnol* 1993; 4: 526-30.

Barbet AF, Anderson LW, Palmer GH, McGuire TC. Comparison of protein synthesized by two different isolates of *Anaplasma marginale*. *Infect Immun* 1983; 40: 1068-74.

Burioni R, Plaisant P, Bugli F, Delli Carri V, Clementi M, Fadda G. A vector for the expression of recombinant monoclonal Fab fragments in bacteria. *J Immunol Methods* 1988; 217: 195-9.

Burton DR. Phage display. *Immunotechnology* (Amsterdam) 1995; 1: 87-94.

Casalvilla R, Duenas M, Ayala M, *et al*. A bacterial single-chain Fv antibody fragment that inhibits binding of its parental anti-E-selectin monoclonal antibody to activated human endothelial cells. *J Biotechnol* 1999; 72: 1-12.

Chapman PA, Siddons CA. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from cases of bloody diarrhoea, non-bloody diarrhoea and asymptomatic contacts. *J Med Microbiol* 1996; 44: 267-71.

Chapman PA, Malo AT, Siddons CA, Harkin M. Use of commercial enzyme immunoassays and immunomagnetic separation systems for detecting *Escherichia coli* O157 in bovine fecal samples. *Appl Environ Microbiol* 1997; 63: 2549-53.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate phenol-choroform extraction. *Anal Biochem* 1987; 162: 2957-63.

Choi KS, Nah JJ, Choi CU, *et al*. Monoclonal antibody-based competitive ELISA for simultaneous detection of rinderpest virus and peste des petits ruminant virus antibodies. *Vet Microbiol* 2003; 96: 1-16.

Feld NC, Qvist P, Ahrens P, Friis NF, Meyling A. A monoclonal blocking ELISA detecting serum antibodies to *Mycoplasma hyopneumoniae*. *Vet Microbiol* 1992; 30: 35-46.

Fukushima H, Hoshina K, Gomyoda M. Selective isolation of eae-positive strains of shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* 2002; 38: 1684-7.

Gavilondo JV, Larrick JW. Antibody engineering at the millennium. *BioTechniques* 2000; 29: 128-49.

Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other

enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* 1991; 13: 60-98.

Griffin PM, Mead PS, Sivapalasingam S. *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*. In: Infection of gastrointestinal tract, 2nd ed. Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL, eds. New York, NY: Lippincott Williams & Wilkins, 2002: 627-42.

Horton RM. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol Biotechnol* 1995; 3: 93-9.

Huse WD, Sastry L, Iverson SA, *et al*. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 1989; 246: 1275-81.

Kim MS, Doyle MP. Dipstick immunoassay to detect enterohemorrhagic *Escherichia coli* O157:H7 in retail ground beef. *Appl Environ Microbiol* 1992; 58: 1764-7.

Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256: 495-7.

Marx U, Embleton MJ, Fischer R, *et al*. Monoclonal antibody production. The report and recommendations of ECVAM Workshop 23. ATLA 1997; 25: 121.137.

McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990; 348: 552-4.

Mori Y, Hamaoka T, Sato S. Use of monoclonal antibody in an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *Mycoplasma hyopneumoniae*. *Israel J Med Sci* 1987; 23: 657-62.

Nagesha HS, Wang LF, Shiell B, Beddome G, White JR, Irving RA. A single chain Fv antibody displayed on phage surface recognizes conformational group-specific epitope of bluetongue virus. *J Virol Methods* 2001; 91: 203-7.

Nataro JP, Kaper JP. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; 11: 142-201.

O'Brien AD, La Veck GD. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. *Infect Immun* 1983; 40: 675-83.

Paton AW, Paton JC. Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for stx1, stx2, eae, ehxA, and saa. *J Clin Microbiol* 2002; 40: 271-4.

Pearson TW, Pinder M, Roelants GE, *et al*. Methods for derivation and detection of anti-parasite monoclonal antibodies. *J Immunol Methods* 1980; 34: 141-54.

Plückthun A, Skerra A. Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. *Meths Enzymol* 1989; 178: 497-515.

Pyle BH, Broadway SC, McFeters GA. Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Appl Enivron Microbiol* 1999; 65: 1966-72.

Riley LW. The epidemiologic, clinical, and microbiological features of hemorrhagic colitis. *Annu Rev Microbiol* 1987; 41: 383-407.

Riley LW, Remis RS, Helgerson SD, *et al*. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 1983; 308: 681-5.

Shelton DR, Van Kessel JS, Wachtel MR, Belt KT, Karns JS. Evaluation of parameters affecting quantitative detection of *Escherichia coli* O157 in enriched water samples using immunomagnetic electrochemiluminescence. *J Microbiol Meths* 2003; 55: 717-25.

ShengFeng C, Suen LP, Xin TW, Feng WG. Construction, expression, purification, refold and activity assay of a specific ScFv fragment against foot and mouth disease virus. *Vet Res Commun* 2003; 27: 243-56.

Sherman P, Soni R, Yeger H. Characterization of flagella purified from enterohemorrhagic, vero cytotoxin-producing *Escherichia coli* serotype O157:H7. *J Clin Microbiol* 1988; 26: 1367-72.

Thomas A, Jiggle B, Smith HR, Rowe B. The detection of vero cytotoxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 in faecal specimens using polymerase chain reaction gene amplification. *Lett Appl Microbiol* 1994; 19: 406-9.

Towbin H, Gordon J. Immunoblotting and dot immunobinding: current status and outlook. *J Immunol Meth* 1984; 72: 313-40.

Waghela SD, Melendy D, Cruz D, Wagner G. Antigenic analysis of *Anaplasma marginale* grown in bovine erythrocytes co-cultured with bovine endothelial cells. *Vet Parasitol* 2000; 94: 133-9.

Westphal O, Jann K. Bacterial lipopolysaccharide: extraction with phenol-water and further applications of the procedure. *Meth Carbohyd Chem* 1965; 5: 83-91.

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. *Annu Rev Immunol* 1994; 12: 433-55.

Wright DJ, Chapman PA, Siddons CA. Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food sample. *Epidemiol Infect* 1994; 113: 31-9.

Zhou B, Wirsching P, Janda KD. Human antibodies against spores of the genus *Bacillus*: a model study for detection of and protection against anthrax and the bioterrorist threat. *Proc Natl Acad Sci USA* 2002; 99: 5241-6.