ISOLATION BY PHAGE DISPLAY OF SINGLE-CHAIN Fv ANTIBODIES AGAINST *Escherichia coli* K99 FIMBRIAE

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Single-chain antibodies (scFv) are small, recombinant proteins in which variable domains from immunoglobulin heavy and light chains are physically linked with a flexible peptide. They are monovalent and lack the heavy chain constant domains for activation of complement and binding to cellular receptors. However scFvs can possess biological activity by binding to and inhibiting the action of their molecular targets. ScFvs can be rapidly isolated from recombinant libraries by phage display. In this study, we have isolated and characterised scFvs against the K99 colonisation factor of an enterotoxigenic strain of *Escherichia coli*.

K99 fimbriae were isolated from B41, a bovine ETEC strain, and purified by ion-exchange chromatography. The material was coated onto plastic immunotubes and two synthetic scFv libraries screened for binders by standard phage display methods. Six scFvs that bound strongly to isolated K99 fimbriae in ELISA were expressed in *E. coli* (HB2151) and purified by nickel-chelating chromatography. Purified scFvs were then used in immunofluorescence microscopy to study their binding to the surface of B41 bacteria. The recognition of fimbriae was also investigated by electron microscopy using immunogold reagents. It was of particular interest to test if any of the anti-K99 scFvs could inhibit the binding of fimbriae to their mammalian receptors. This was tested in haemagglutination assays. As expected, several of the anti-K99 antibodies have shown the inhibitory activity.

Our studies illustrate how recombinant antibody technology can be applied to the study of pathogenesis for infectious disease.

Keywords: Recombinant antibodies, scFv, Phage display, K99

Introduction

The K99 colonisation factor is a well-characterised virulence determinant expressed by strains of enterotoxigenic strains of *Escherichia coli* important to the beef and dairy industries. Antibodies against these fimbrial structures, passively acquired by the calf from the dam have protective efficacy. Anti-K99 antibodies are also of importance in the diagnosis of ‘scours’ or calf diarrhoea and can be administered therapeutically. Given our interest in the application of recombinant antibody technology to livestock infection, we chose K99 as a target for this study. Our aims were to isolate and characterise scFv antibodies against K99 using phage display.

Purification of K99 fimbriae

*Escherichia coli* B41, a clinical isolate that expresses K99 fimbriae, was obtained from the Central Veterinary Laboratory, Surrey, UK. Bacteria were propagated on sheep blood agar and inoculated to Minca liquid medium for overnight culture. Bacteria were harvested by centrifugation, resuspended in phosphate buffer containing urea and fimbriae were released by heating the suspension to 65°C. After the removal of bacteria by centrifugation, fimbriae were precipitated from the supernatant with 60% saturated ammonium sulphate, centrifuged, and redissolved in urea solution. The buffer was exchanged with Tris pH 7.0 using Amicon Ultra 4 centrifugal filters and samples then loaded to a HiTrap SP XL column (Amersham Biosciences). By step-wise elevation of the sodium chloride concentration, contaminating proteins were eluted before recovery of the K99 fimbrial subunits. Fractions were pooled and concentrated using Ultra 4 filter units before dialysis into PBS.

Analysis of the purified material by SDS PAGE showed it to contain a single protein of the molecular weight expected for the major fimbrial subunit (Figure 1). The purified material
successfully agglutinated latex beads coated with anti-K99 monoclonal antibody (Fimbrex test, CVL, Surrey). It also agglutinated sheep erythrocytes and was recognised by polyclonal and monoclonal anti-K99 antibodies in ELISA.

**Figure 1.** SDS PAGE analysis of purified K99. M – marker

**Isolation of anti-K99 scFv antibodies**
The Tomlinson I and J libraries were obtained from the MRC Centre for Protein Engineering, Cambridge, UK. Each comprises in excess of 1 x 10^8 synthetically diversified scFv antibodies, constructed upon human VH and Vk frameworks that are well-expressed in bacteria. Phage displaying each library were produced by superinfection with KM13 helper phage. The stocks were titred by their ability to transduce *E. coli* TG1 to ampicillin resistance. Around 1 x 10^13 phage from each library were then added to immunotubes (Nunc) coated with K99 at 20 mg/ml. After incubation and rigorous washing, phage able to interact with the target were eluted by trypsinisation. Recoveries were assessed by infection into *E. coli* TG1 and plating on TYE agar containing ampicillin before amplification by superinfection and preparation of fresh phage stocks for the next round of selection. Three rounds of panning were carried out with immunotubes coated with a lower concentration of K99 (10 mg/ml).

Progress of panning was judged from the input and output titres of phage at each round of selection (Table 1). Clones were also picked at random after each round of panning, phage prepared, and their recognition of K99 assessed using ‘monoclonal phage ELISA’ (Figures 2 and 3). Results showed that selection successfully enriched for specific anti-K99 scFvs, round on round.

**Table 1. Phage titres during panning for anti-K99 scFvs**

<table>
<thead>
<tr>
<th>Round</th>
<th>Tomlinson I</th>
<th>Tomlinson J</th>
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<tbody>
<tr>
<td></td>
<td>Input</td>
<td>Output</td>
</tr>
<tr>
<td>Round 1</td>
<td>1.3 x 10^{13}</td>
<td>4.4 x 10^5</td>
</tr>
<tr>
<td>Round 2</td>
<td>1.4 x 10^{12}</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>Round 3</td>
<td>1.4 x 10^{12}</td>
<td>3.1 x 10^8</td>
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**Properties of anti-K99 scFvs**
Numerous scFv clones that emerged from round 3 of selection showed reaction with K99 in ELISA when displayed at the phage surface (Figure 3) and when expressed as soluble protein.
Two criteria were applied to choose clones for closer analysis: higher than average reaction with K99 in ELISA; scFv sequences that were representative of the major groupings observed. On this basis, 4 clones from library I and 2 from library J were selected. The antibodies were expressed as soluble scFv in *E. coli* HB2151 and purified from the culture supernatant by nickel chelation chromatography. In all cases, a single protein of about 30 kDa was isolated although yields were variable. The ability of these scFvs to recognise K99 fimbriae at the bacterial surface was tested using immunofluorescence microscopy and immunogold techniques for electron microscopy. The binding of polyclonal (Figure 4A) and scFv antibodies (Figure 4B, 4C) to fimbriae was evident and showed specificity (Figure 4D) when B41 bacteria were grown at 37°C. At 18°C, K99 fimbriae were not expressed and antibody binding was not observed (Figure 4E). Mixing *E. coli* B41 with sheep erythrocytes triggered haemagglutination, a reaction that was dependent upon culture of bacteria at 37°C (Figure 5). Haemagglutination could be inhibited by pre-incubation of bacteria with high dilutions of polyclonal anti-K99 antibodies. Purified scFvs were tested in this assay revealing that some possessed inhibitory activity (Table 2).

**Figure 4.** Detection of binding of antibodies to K99 by immunogold EM. *E. coli* B41 grown at 37°C (A, B, C and D) or 18°C (E) were fixed and incubated with polyclonal anti-K99 antibodies (A and E), scFvs F7 (B) or F10 (C) against K99, or an anti-ubiquitin scFv (D). Five-nm colloidal gold particles conjugated to Protein A were then added. The grids were then prepared for EM by a negative staining method.

**Figure 5.** Haemagglutinating activity of *E. coli* B41. Bacteria were grown at overnight at 37°C (row A) or 18°C (row B) and serial dilutions prepared prior to addition of sheep erythrocytes. B41 – undiluted bacteria (37°C); NB – no bacteria.

**Table 2.** Properties of anti-K99 scFv antibodies.

Purified scFvs were adjusted to equal protein concentrations and tested for anti-haemagglutinating activity by incubation at serial dilutions with *E. coli* B41 before addition of sheep erythrocytes. Titre was defined as the highest dilution that prevented red cell agglutination. ND – not determined. scFvs were also tested in ELISA against K99 at equal protein concentrations. Assay results were banded into high (H), moderate (M) and low (L) reactivity on a relative basis.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Tomlinson library I</th>
<th>Tomlinson library J</th>
<th>Polyclonal anti-K99</th>
</tr>
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<tbody>
<tr>
<td>Anti-haemagglutinating titre</td>
<td>8</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Anti-K99 ELISA activity</td>
<td>L</td>
<td>M</td>
<td>L</td>
</tr>
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Conclusions
Past experience has taught us that library quality is the single most important factor in work in this area. In this regard, the Tomlinson libraries proved excellent resources from which to isolate anti-K99 scFvs. Numerous anti-K99 scFvs were isolated from which we selected 6 for detailed analysis. Whilst the immunochemical properties of these clones may be of diagnostic value, the ability of some to block K99-mediated haemagglutination was of particular interest. We have set up an in vitro model to assess the binding of *E. coli* to isolated intestinal villi from young calves. We aim to use this system to further evaluate the ability of scFvs to block the biological function of K99 fimbriae. As scFv antibodies are monovalent, future work will also determine if anti-colonisation activity is attributable to binding to the receptor-recognition site on the major subunit of the adhesin.

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