Selection of an scFv phage antibody that recognizes bluetongue virus from a large synthetic library and its use in ELISAs to detect viral antigen and antibodies

W. VAN WYNGAARDT and D.H. DU PLESSIS

Immunology Division, Onderstepoort Veterinary Institute, Private Bag X5 Onderstepoort, 0110 South Africa

ABSTRACT

W. VAN WYNGAARDT & D.H. DU PLESSIS. 1998. Selection of an scFv phage antibody that recognizes bluetongue virus from a large synthetic library and its use in ELISAs to detect viral antigen and antibodies.*Onderstepoort Journal of Veterinary Research,* 65:125–131

A filamentous phage library displaying a vast repertoire of synthetic single chain fragment variable (scFv) antibody fragments was subjected to affinity selection on purified bluetongue virus (BTV) particles. After four rounds of selection and amplification, 73 out of a total of 90 fusion phage clones tested were found to bind to purified BTV in ELISA. One of these, the clone producing the highest ELISA signal, was selected for an investigation of its potential as an immunodiagnostic reagent. The binding of this phage antibody (designated A12) could be inhibited by free virus and by antibodies in immune serum. Inhibition with antibodies in guinea-pig sera suggested that it recognized an antigenic region on BTV that was similar on at least 10 different BTV serotypes. A sandwich ELISA utilizing antibody A12 was capable of detecting approximately 60 ng of purified BTV.

Keywords: Bluetongue virus, ELISA, phage antibodies, synthetic library

INTRODUCTION

Bluetongue virus (BTV) represents the prototype of the *Orbivirus* genus in the Reoviridae family. The mature particle comprises 10 monocistronic dsRNAs enclosed by a double-shelled capsid consisting of seven different proteins; VP1, VP3, VP4, VP6 and VP7 make up the core while VP2 and VP5 are located on the outer capsid layer. The virus is transmitted by midges of the genus *Culicoides* and causes an economically important disease of sheep in southern Africa. There are at least 24 serotypes of BTV which are defined by their ability to be neutralized by type-specific antisera (for reviews see Roy 1989, 1992; Gould & Hyatt 1994).

Monoclonal antibodies (Mabs) have played an important role in BTV diagnosis and research. For example, they have been used in assays for detecting virus-specific antibodies in animal sera (Andersen 1984; Lunt, White & Blacksell 1988; Afshar, Heckert, Dulac, Trotter & Myers 1995; Zhou & Huang 1995), in research into BTV antigenicity and structure (Appleton & Letchworth 1983; Letchworth & Appleton 1983; Hyatt & Eaton 1988; Wang, Du Plessis, White, Hyatt & Eaton 1995) and in the development of alternative vaccines (Lin, Zhou & Heckert 1996). These studies have so far all depended upon Mabs produced by classical hybridoma technology (Köhler & Milstein 1975).

Phage display (Smith 1985; McCafferty, Griffiths, Winter & Chiswell 1990; Scott & Smith 1990) now provides an alternative means of obtaining antibodies to viral (Harper, Kerschbaumer, Ziegler, Macintosh, Cowan, Himmler, Mayo & Torrance 1997) and other antigens (Vaughan, Williams, Pritchard, Osbourn, Pope, Earnshaw, McCafferty, Hodits, Wilton & Johnson 1996). In this approach, antigen-specific antibodies are selected (Parmley & Smith 1988) from a filamentous

Accepted for publication 7 April 1998—Editor

phage library displaying a vast repertoire of different antibody specificities. While it is possible to access the immunoglobulin genes of an immune animal for phage display expression (Clackson, Hoogenboom, Griffiths & Winter 1991), the introduction of large naïve (Vaughan *et al.* 1996) or synthetic (Nissim, Hoogenboom, Tomlinson, Flynn, Midgley, Lane & Winter 1994) repertoires has effectively eliminated the requirement for immunization. In addition, phage display offers the possibility of rapidly obtaining antibodies of the desired specificity, the ability to manipulate affinities *in vitro* (Marks, Griffiths, Malmqvist, Clackson, Bye & Winter 1992; Neri, Carnemolla, Nissim, Leprini, Querzè, Balza, Pini, Tarli, Halin, Neri, Zardi & Winter 1997) and the potential for using a single library as a source of antibodies directed against many different antigens (Nissim *et al.* 1994; Vaughan *et al.* 1996).

The phage display antibody fragment library constructed by Nissim and co-workers (1994) has been found to be a ready source of recombinant antibodies that function well in commonly used immunoassays. This library ("Nissim library") is in the single chain format and consists of fusion phages displaying a diverse repertoire of *in vitro* rearranged human V_H genes, each containing a randomized synthetic CDR3 of 4–12 residues in length. The rearranged V_H genes are paired with a single unmutated V_L chain gene segment and are expressed as part of the bacteriophage minor protein, pIII. This library has yielded phage antibodies to a variety of different antigens, e.g. keyhole limpet haemocyanin, maltose binding protein, human tumour suppressor protein p53, crototoxin and potato leafroll luteovirus (Nissim *et al.* 1994, Harper, *et al.* 1997; Lafaye, Choumet, Demangel, Bon & Mazié 1997). In the light of these successful applications, we wished to establish whether we could select a BTV-binding phage antibody from the Nissim library and if so, whether such an antibody could provide a possible alternative to Mabs produced by classic methods for use in immunoassays aimed at detecting either the virus or its antibodies.

MATERIALS AND METHODS

Antigens and antisera

BTV serotype 10 (BTV10) virus stock and guinea pig antisera raised against crude virus suspensions that had been adsorbed onto aluminium hydroxide were obtained from the International Office Of Epizootics (OIE) World Reference Centre for orbiviruses at the Onderstepoort Veterinary Institute. Virus was propagated in baby hamster kidney (BHK) cells and purified by sucrose density-gradient centrifugation (Huismans, van der Walt, Cloete & Erasmus 1987). It was used at a concentration of 10 µg/mℓ in ELISA for detecting BTV binding phage antibodies. Maltose binding protein (MBP) was obtained from New England Biolabs (MBP2 Cat. No. 800-44S) and an scFv phage antibody specific for MBP (Nissim *et al.* 1994) was provided by the MRC Centre for Protein Engineering, Cambridge, U.K.

Library amplification and rescue of phage particles

A human synthetic single chain fragment variable (scFv) phage display library with a diversity of $>1x10^8$ clones (Nissim *et al.* 1994) was supplied by the Centre for Protein Engineering in Cambridge, (U.K.). It was amplified and phage particles were rescued using M13K07 helper phage essentially as described by Marks, Hoogenboom, Bonnert, McCafferty, Griffiths & Winter (1991). A volume of 100 mℓ 2 x TY (16 g tryptone, 10 g yeast extract and 5 g NaCl dissolved in double distilled deionized water) supplemented with 100 µg/mℓ ampicillin and 2% w/v glucose (2 x TY A/G) was inoculated with $2,8 \times 10^9$ bacteria from the library stock. The bacteria were grown at 37°C in a shaking incubator at 250 rpm until the A_{600} reached 0,5. A 20 mℓ volume of this culture was infected with $1,6 \times 10^{11}$ M13KO7 helper phages (helper phage:bacteria = 20:1) and incubated for 30 min at 37°C without shaking. After centrifugation at 2 000 x *g* for 10 min, the infected cells were resuspended in 600 mℓ 2 x TY that contained 100 µg/mℓ ampicillin and 25 µg/mℓ kanamycin (2 x TY A/K) and shaken at 250 rpm overnight at 30°C. Bacteria were removed by centrifugation at 10 800 x *g* for 10 min. Phage particles were precipitated by adding 1/5 volume of 20% w/v polyethylene glycol 6000 in 2,5M NaCl and resuspended in 80 mℓ phosphate buffered saline pH 7,4 (PBS). Before storage at –70°C, the precipitation was repeated and the final pellet was resuspended in 8 mℓ PBS containing 15% (v/v) glycerol. The resulting phage particles were used in the first selection round ("panning"). The same protocol was used to prepare phage particles for consecutive rounds except that 5 mℓ bacterial cultures (A_{600} = 0,5) were rescued and pellets were resuspended in 5 volumes 2 x TY A/G. Furthermore, phage particles were precipitated only once between each panning.

Individual phage clones for ELISA screening were rescued in the wells of a microtitre plate by transferring inocula from bacterial colonies to a sterile 96 well flat-bottom immunoplate (Nunc Polysorp) containing 100 µℓ/well 2 x TY A/G. Bacteria were grown overnight at 30°C and 250 rpm. The next day, a 96-well inoculation device (Sigma: Cat. no. R-2508) was used to transfer two inocula of approximately 1 µℓ each from the master plate to a fresh plate that contained 150 µℓ 2 x TY A/G per well. After 2,5 h incubation at 250 rpm and 37°C, a 50 µℓ volume 2 x TY A/G that contained 2×10^9 pfu/mℓ M13KO7 was added to each well. The plate was incubated at 37°C for 30 min without shaking. After centrifugation for 10 min at 600 x *g* the supernatant fluids were removed and replaced with

150 µℓ of 2 X TY A/K. The plate was incubated overnight at 30 °C and 250 rpm.

For all other ELISAs, phage particles were produced by incubating a 5 mℓ volume of 2xTY/AG inoculated with 50 µℓ of an overnight bacterial culture infected with phagemids and 5 x 10^8 pfu/mℓ M13KO7 helper phages for 2 h at 37 °C and 250 rpm. After centrifugation, the bacterial pellet was resuspended in 10 mℓ 2 x TY/AK and incubated overnight at 30 °C and 250 rpm. Prior to use, bacterial supernatant fluids were prepared by centrifugation for 15 min at 2 000 x *g*.

Selection of scFv phage antibodies

The protocol used was modified from methods described by Marks *et al.* (1991). Four consecutive rounds of panning were carried out in immunotubes (Nunc; Polysorp) coated with purified BTV10 (10 µg/ℓ) in PBS for 2 h at room temperature (RT). After two washes with PBS, the tubes were incubated for 1 h at RT with 2% w/v skimmed milk powder (MP) in PBS. The wash step was repeated and 10^{12}–10^{13} phage particles, pre-incubated for 30 min at RT in 4 mℓ 2% w/v MP, were transferred to the immunotube. The tube was incubated at RT on a rotating turntable for 30 min and then left undisturbed for a further 1,5 h. Tubes were washed 20 times with PBS containing 0,1 % v/v Tween 20 (PBS/T) and a further 20 times with PBS by pouring the wash buffer in and out immediately. Bound phages were eluted by adding 1 mℓ 100 mM triethylamine pH 12 (TEA) followed by 10 min incubation on a rotary turntable. The eluate was immediately neutralized in a microfuge tube containing 0,5 mℓ 1M pH 7,4 Tris/HCl and the immunotube was rinsed with a 200 µℓ volume of the same buffer to neutralize any residual TEA. A 1 mℓ aliquot of the neutralized eluate was used to infect 9 mℓ of logarithmic phase *Escherichia coli* TG1 cells (Pharmacia) in 2 x TY medium for 30 min at 37 °C. After elution, the immunotube was also incubated for a further 30 min at 37 °C with 4 mℓ of logarithmic phase TG1 cells to promote recovery of phages not released by the TEA treatment. The latter two cell suspensions were combined and centrifuged at 2 000 x *g* for 10 min. The cell pellet was resuspended in 1 mℓ 2 x TY and 500 µℓ volumes were plated on two 15 cm diameter TYE nutrient agar (15 g agar, 8 g NaCl, 10 g tryptone, 5 g yeast extract/litre water) plates containing 2% w/v glucose and 100 µg/mℓ ampicillin. The plates were incubated overnight at 30 °C. After four such rounds of selection, individual clones were picked and phages were rescued as described above. Soluble fragments were prepared by glucose withdrawal to induce production of soluble fragments (De Bellis & Schwartz 1990).

Indirect ELISA

Immunoplate wells were coated by overnight incubation at 4 °C with 50 µℓ per well of the appropriate antigen. Blocking was by incubation for 2,5 h at 30 °C with 300 µℓ per well of 2% MP in PBS. All washes were done with PBS/T using an automated microtitre plate washer (Anthos, Austria). Volumes of 50 µℓ of phage antibody-containing supernatant fluids (see microtitre plate rescue protocol) diluted 1:1 with PBS containing 0,2% Tween-20 and 4% (w/v) MP were added to the washed antigen-coated wells and incubated for 90 min at 30 °C. Subsequent to a further wash, a 50 µℓ volume of anti-M13KO7 IgG conjugated to horseradish peroxidase (Pharmacia 27-9402A) diluted 1/5000 in PBS-MP was added and incubated at 30 °C for 90 min. After a final wash, 50 µℓ per well of chromogen consisting of 1 mg/mℓ *o*-phenylene diamine and 0,5 µℓ/mℓ of a 30% (v/v) hydrogen peroxide solution in 0,1 M citrate buffer, pH 4,5 was added. Absorbance was monitored at either 450 nm or 492 nm (reaction stopped by adding an equal volume of 2N H_2SO_4) using an EAR 400 AT microtitre plate reader (SLT, Austria).

Sandwich ELISA

Microtitre plate wells were coated for 2 h at 30 °C with 50 µℓ/well of a 10 µg/mℓ *Staphylococcus aureus* Protein A (ZYMED cat. no. 10-1100) solution in PBS. Blocking was for 1 h at 30 °C with 300 µℓ per well of 2% (w/v) skimmed milk powder in PBS. An anti-BTV10 serum prepared in guinea-pigs and diluted 1/30 in 2% w/v skimmed milk powder was used as trapping antibody. The plate was coated for 1 h at 30 °C with 50 µℓ per well with this antibody. After 3 washes with PBS/T the plate was coated for 1 h at 30 °C with 50 µℓ volumes 2% MP in PBS that contained various concentrations of purified BTV10. The remaining steps were identical to those described for the indirect ELISA except for a reduction in incubation times from 90–60 min.

RESULTS

Selection of BTV-binding phage antibodies

BTV binders were selected by subjecting the Nissim phage antibody library to four rounds of panning on an affinity matrix consisting of purified virus adsorbed to a polystyrene surface. During each round of selection, phages were quantified as colony-forming units to allow calculation of the input/output ratios. The relative number of phages recovered after panning increased with each consecutive round, demonstrating that a higher proportion of antigen binders was present in the input of each subsequent round of selection. Enrichment factors (enrichment factor = input:output for selection round *n* / input:output for round *n-1*) of 17, 21 and 26-fold were obtained after selection rounds 2, 3 and 4, respectively. After the fourth round, 90 phage clones were picked and individually tested in ELISA for binding, both to BTV and

to the milk powder components used as blocking agent during selection. Although 86 of the clones yielded ELISA signals on BTV that were at least double the highest signal obtained on MP (A_{450} = 0,031), an absorbance value of 0,2 was arbitrarily chosen as a cut-off value for putative BTV binders. Based on this criterion, a total of 73 bacterial clones produced recombinant phage antibodies that bound to BTV. Their A_{450} values in ELISA varied from 0,210 to 1,437. The clone which gave the highest signal in the screening assay was chosen for further investigation. It was found to bind to BTV, but not to BHK cell homogenate, MBP or to MP proteins. In addition, controls consisting of the native phagemid vector pHEN1 (Hoogenboom, Griffiths, Johnson, Chiswell, Hudson & Winter 1991) and a MBP binding scFv phage antibody did not recognize BTV (Fig.1). This phage antibody, designated A12 from its position in the microtitre plate, was further confirmed to be a BTV-binder by pre-incubating with free antigen prior to ELISA. In this experiment, phage antibody-containing culture supernatants were incubated with various concentrations of purified virus, mixed with an equal volume of of PBS containing 4% MP and 0,2% Tween 20 (i.e. double normal strength), and then added to antigen-coated microtitre plate wells. The highest concentration of BTV that was tested (40 µg/mℓ) reduced the ELISA signal produced by phage antibody A12 to approximately 25% of its initial value (Fig.2).

The phage antibody A12 was also tested in indirect ELISA in the non-fusion phage format to establish whether the binding properties of soluble antibody fragments were comparable with those of the phage displayed scFv fragments. It was, however, not possible to demonstrate binding to BTV by these fragments (not shown).

Detection of BTV antibodies by inhibition ELISA

In order to be evaluated in an immunoassay for detecting virus antibodies, it was necessary to ascertain whether the binding of the synthetic phage antibody A12 could be inhibited by BTV antibodies in an immune serum. Dilutions of a guinea-pig antiserum directed against BTV-10 particles were made in PBS containing 2% MP and pre-incubated for 1 h at 30 °C together with immobilized virus in the wells of a microtitre plate. Upon addition of antibody A12 and subsequent detection with an enzyme-labelled anti-phage antibody, it was found that the homologous antiserum could be diluted by as much as 810 times and still inhibit the final ELISA reading by more than 45% (Fig. 3). Antisera directed against nine other BTV serotypes were also each tested at a dilution of 1/30 using the same experimental protocol. All caused a reduction in ELISA signal when compared with a non-immune serum. Inhibition values ranging from 44% to 76% were obtained (Fig. 4). These differences probably reflect titre and avidity variations between the different competing antisera and not true serological relationships. The inhibition values were calculated as follows:

Percentage inhibition = (Reduction in absorbance after incubating antigen in presence of immune serum/Absorbance after incubating antigen in presence of pre-immune serum) x 100

Detection of BTV by sandwich ELISA

Filamentous phages have approximately 2700 copies of the major structural protein pVIII (Newman, Swinney & Day 1977). Since pIII is involved in the interaction with the antigen, a large number of enzyme-labelled anti-bacteriophage antibodies can potentially bind to the capsid of each bound fusion phage. To test whether phage antibody A12 could be used as a second antibody in a sandwich enzyme immunoassay, the wells of a microtitre plate were first coated with *S. aureus* Protein A to selectively trap guinea-pig IgG from a BTV antiserum. The immobilized immunoglobulins were in turn used to capture virus particles from suspension. To detect the trapped virus particles, phage antibody A12 was then added, followed by enzyme-labelled anti-phage antibodies. Fig. 5 shows that BTV could be effectively detected and quantitated by this assay. As little as 1,25 µg/mℓ of purified BTV10 particles gave an ELISA signal of at least twice that of the background reading. This is equivalent to 62,5 ng of virus per microtitre plate well.

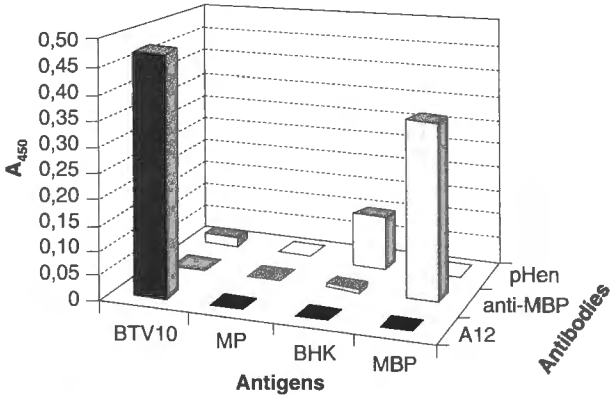

FIG.1 Indirect ELISA showing specificity of phage antibody A12 for BTV. The antibody was tested for binding to bluetongue virus (BTV10), milk powder (MP), maltose binding protein (MBP) and baby hamster kidney cell antigens (BHK). A recombinant anti-MBP phage antibody was used as a positive ELISA control. The negative control was the native phage display vector (pHEN1; Hoogenboom, Griffiths, Johnson, Chiswell, Hudson & Winter 1991)

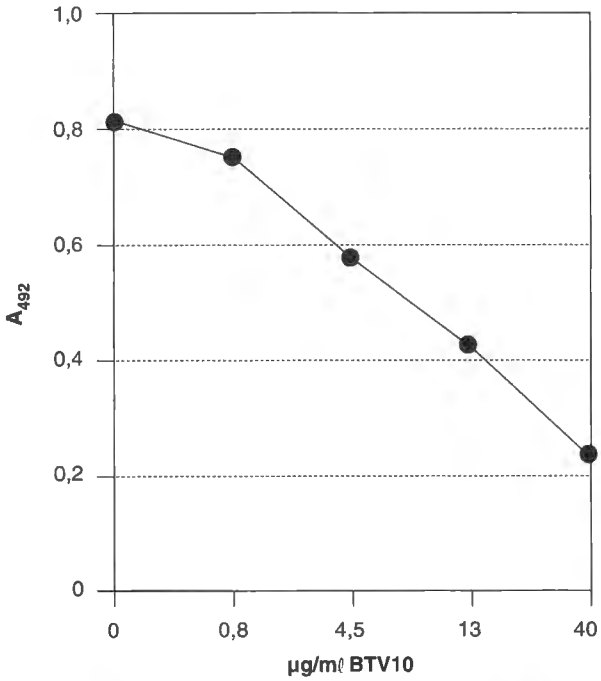

FIG. 2 ELISA showing inhibition by free virus of the binding of phage antibody A12 to immobilized BTV10

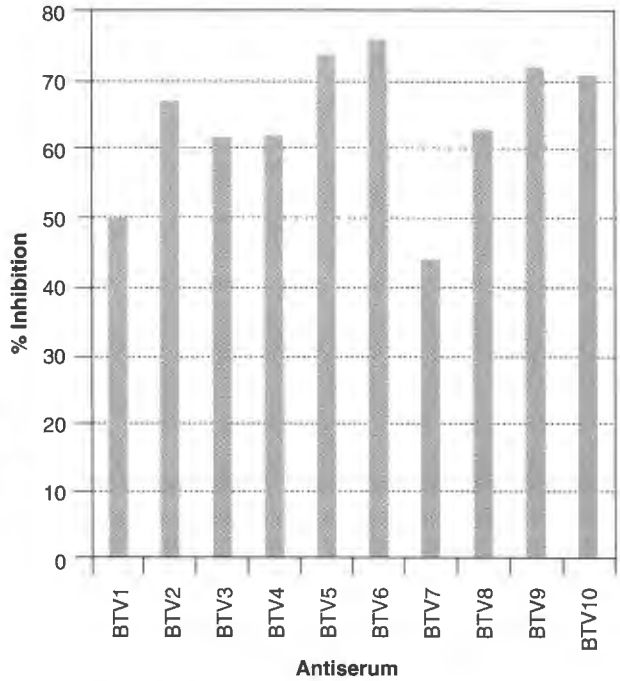

FIG. 4 Inhibition ELISA showing the reduction in signal produced by phage antibody A12 after pre-incubation of immobilized purified BTV10 with anti-BTV guinea-pig sera directed against 10 different BTV serotypes (BTV1-10). Percentage inhibition was calculated by comparison with a pre-immune serum

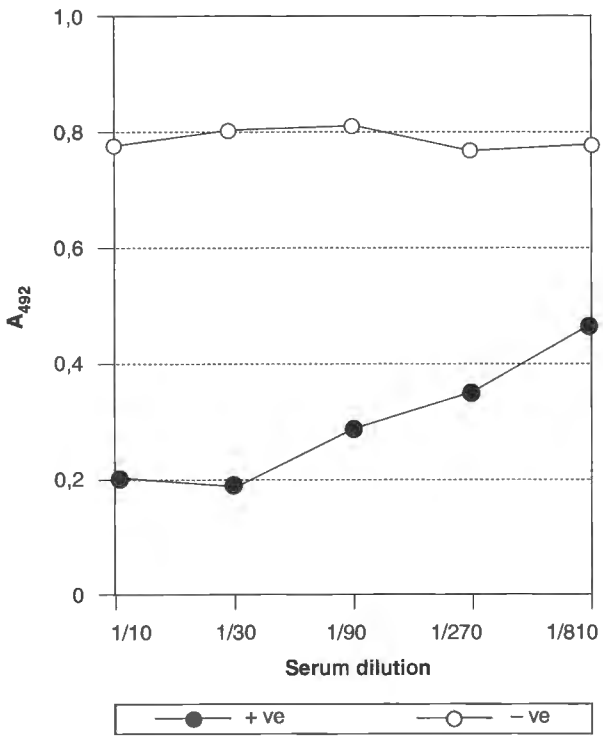

FIG. 3 Inhibition ELISA illustrating the ability of various dilutions of anti-BTV10 guinea-pig serum (+ve) to inhibit the binding of phage antibody A12 to purified BTV10. The negative control (-ve) was a guinea-pig pre-immune serum

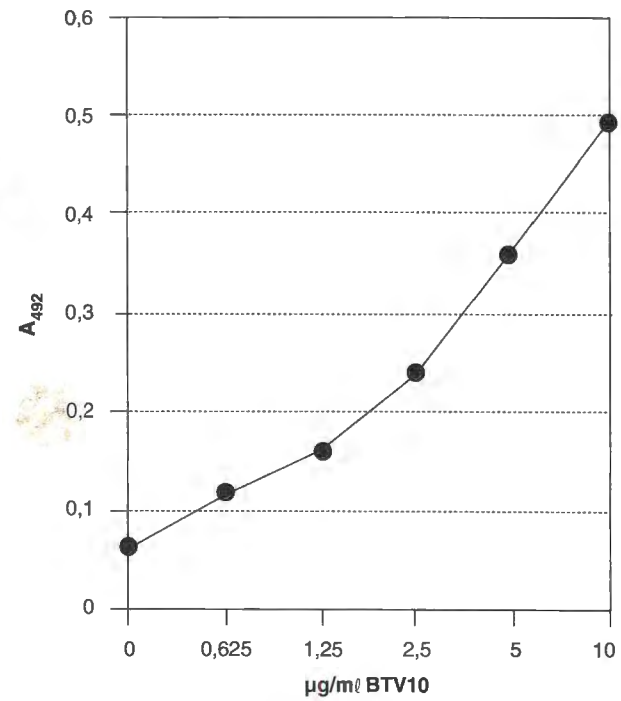

FIG. 5 Detection of purified BTV10 by sandwich ELISA using guinea-pig anti-BTV10 immunoglobulins for antigen capture and phage antibody A12 for detection

DISCUSSION

A panel of BTV-binding scFv phage antibodies was selected from a large synthetic phage display library by panning on immobilized BTV. After four rounds of panning and amplification, 86 phage clones that reacted with BTV to a greater or lesser extent in ELISA were obtained. Of the 73 phages finally adjudged to represent BTV binders, antibody A12 was considered on the basis of its ELISA reactivity to be suitable for further investigation as an immunodetection reagent. In addition to its binding to immobilized virus in indirect ELISA (Fig. 1), its ability to recognize BTV was shown by inhibiting with different concentrations of free virus. Since this exercise results in a calibration curve if purified virus is used (Fig. 2), it may be feasible to use such an approach to determine the amount of BTV in impure preparations such as cell culture suspensions. The binding of antibody A12 to immobilized virus was also efficiently inhibited by guinea-pig antiserum to the homologous serotype 10 and to nine other BTV serotypes (Fig. 3 and 4), indicating that the phage antibody recognized a region on the viral capsid that was likely to be structurally conserved on each of the different serotypes. Other than general surface accessibility, however, there is no reason why the "epitope" recognized by such a phage-displayed antibody fragment should necessarily correspond to a true antigenic determinant as recognized by the immune system of an animal. Nevertheless, since the phage antibody was clearly inhibited by the "natural" antibodies in guinea-pig antiserum, it is probable that it did in fact bind to a region of the viral capsid that represented, or was located near to, an authentic epitope. This finding illustrates that despite not having been produced by immunization, the recombinant phage-displayed antibody fragment can be a useful reagent in a competition type immunoassay aimed at detecting viral antibodies in mammalian serum. Since VP2, VP5 and VP7 are all accessible on the virion surface (Verwoerd, Els, De Villiers & Huismans 1972; Hyatt & Eaton 1988), and assuming that capsid integrity is maintained during the ELISA, it seems likely that phage antibody A12 interacted with a structurally conserved area on one or more of these proteins. Because of its antigenic similarity between serotypes (Huismans & Erasmus 1981; Gumm & Newman 1982), it seems likely that VP7 is involved in this interaction.

While the scFv fusion phage antibody A12 bound convincingly to immobilized antigen in indirect ELISA, this was not the case with its corresponding soluble scFv fragments. One explanation for this apparent discrepancy is that each phage particle probably displays more than one antibody fragment, thereby producing a cooperative avidity effect (Nissim *et al.* 1994). This is likely to be particularly significant when binding to a large multivalent antigen such as a virus particle. By contrast, the individual affinities of the soluble fragments for BTV may not have been sufficient to allow them to withstand the repeated washing to which they were subjected in ELISA. Another possibility is that the sensitivity of detection of the fusion phage particles was higher than that of the scFV soluble fragments owing to amplification resulting from the multiple copies of gene VIII which were available to react with the phage antibody-enzyme conjugate that was used for detection. This potential for binding multiple copies of a labelled detection reagent was exploited by using the phage antibody A12 as a second antibody in a sandwich ELISA. In this format, an immobilized mammalian immunoglobulin was used as the first antibody to trap BTV particles from suspension. The resulting immunoassay could detect purified BTV at a concentration of just over 1 µg/mℓ (Fig. 5). As a virus detection method, it offers an alternative to inhibition with free antigen as described above.

To summarize, the work described here has reconfirmed the MRC Nissim library as a viable "single-pot" source of immunochemical reagents and has resulted in the selection of a number of potentially useful new phage antibodies, one of which was shown to be suitable for use in a variety of ELISA formats for detecting BTV or its antibodies. The phage display approach to antibody production may also be able to provide other reagents that could facilitate the development of new BTV diagnostic methods. For instance, because of the vast number of specificities available, an extended examination of the BTV binders in the Nissim library (or other similar repertoire), is likely to identify antibodies that recognize the virus with widely differing binding characteristics. It may therefore be possible to find phage antibodies that specifically recognize only one BTV serotype. Having a series of such antibodies could make it feasible for enzyme immunoassays to replace the virus neutralization methods currently used to routinely identify BTV serotypes. Another advantageous characteristic is that a phage-displayed antibody fragment has its paratope physically linked to its encoding DNA and can therefore be readily subjected to mutagenesis in its CDR. A possible way of exploiting this would be to alter its affinity so that unwanted cross-reactions, e.g. with other serotypes or virus groups, are either abrogated or greatly reduced. The findings reported here could therefore provide a platform on which to base the development of novel immunodiagnostic tests for BTV.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Medical Research Council in Cambridge (U.K.) for providing us with the Nissim library and thank Fiona Sait for handling the correspondence and shipping. We are indebted to Greg Winter for his part in making it possible for us to acquire this valuable resource. The OIE World Reference

Centre for orbiviruses at the Onderstepoort Veterinary Institute is acknowledged for providing BTV and virus antisera. WvW thanks Hennie Hoogenboom and colleagues at CESAME for laboratory experience and for their manual of phage display protocols.

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