New Microbiologica, **35**, 289-294, 2012

A phage display vector optimized for the generation of human antibody combinatorial libraries and the molecular cloning of monoclonal antibody fragments

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SUMMARY

A novel phagemid vector, named pCM, was optimized for the cloning and display of antibody fragment (Fab) libraries on the surface of filamentous phage. This vector contains two long DNA "stuffer" fragments for easier differentiation of the correctly cut forms of the vector. Moreover, in pCM the fragment at the heavy-chain cloning site contains an acid phosphatase-encoding gene allowing an easy distinction of the *Escherichia coli* cells containing the unmodified form of the phagemid versus the heavy-chain fragment coding cDNA. In pCM transcription of heavy-chain Fd/*gene III* and light chain is driven by a single *lac*Z promoter. The light chain is directed to the periplasm by the *ompA* signal peptide, whereas the heavy-chain Fd/coat protein III is trafficked by the *pelB* signal peptide. The phagemid pCM was used to generate a human combinatorial phage display antibody library that allowed the selection of a monoclonal Fab fragment antibody directed against the nucleoprotein (NP) of Influenza A virus.

KEY WORDS: Phagemid vector, Combinatorial antibody library, Phage display, Human monoclonal antibody fragments (mFabs).

Received June 18, 2012 *Accepted* July 02, 2012

INTRODUCTION

Combinatorial phage display vectors represent a powerful tool for the cloning of the vast diversity of the immunological repertoire and for obtaining monoclonal antibodies (Rader *et al.*, 1997). Phage display antibody library selection process largely depends upon various molecular factors such as methods for phage library construction, phage/phagemid vectors, host cells and biopanning process (Barbas *et al.*, 1991; Breitling *et al.*, 1991; Burioni *et al.*, 1997; Bugli *et al.*, 2011, Clementi *et al.*, 2012; Conrad *et al.*, 2005).
Using the phage display technology, monoclonal antibodies against a vast number of antigens have been generated (Bugli *et al.*, 2001; Burioni *et al.*, 1994; Burioni *et al.*, 1998a; Burioni *et al.*, 2002; Burioni *et al.*, 2004; Clementi *et al.*, 2011; De Marco *et al.*, 2012; Kim *et al.*, 2004; Mancini *et al.*, 2009; Williamson *et al.*, 1993; Yu *et al.*, 2009). In particular, monoclonal antibodies against infectious pathogens can find important applications in diagnosis (Desogus *et al.*, 2003; Williamson *et al.*, 1997) and therapy (Burioni *et al.*, 2008; Mancini *et al.*, 2011). Importantly, the possibility of cloning the entire repertoire representing the humoral response against key microbial or viral antigens allows the dissection of the immunoresponse with important implications for the understanding of host-pathogen interplay (Burioni *et al.*, 2009; Perotti *et al.*, 2008) and the development of vaccination and therapeutic strategies (Williamson *et al.*, 1993).
The careful construction of a phage display antibody library is the most important step for the

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success of mAb cloning. Phage display vectors with different features have been generated (Barbas *et al.*, 1991; Burioni *et al.*, 1997; Huse *et al.*, 1989; Kang *et al.*, 1991; Qi *et al.*, 2012) and among all pComb3 and the its optimized version pComb3H (Barbas *et al.*, 1991; Lang *et al.*, 1996; Yu *et al.*, 2009) have been the most used vectors. However, one of the drawbacks of pComb3 is represented by the fact that it has small "stuffers" separating the two couples of enzymes cutting the phagemid at sites where light-chain and the heavy-chain-fragment DNA have to be cloned. Therefore, this aspect does not allow differentiation of the correctly cut form of the vector from the uncorrected one. If using a vector already containing the antibody genes, once again it is not possible to distinguish the vector that accepted the new genes from the one representing the background. In this work we report the construction of a new vector, named pCM, obtained through modification of the commercially available phagemid pBluescript (Stratagene). The pCM vector, while retaining all the desirable characteristics of previously used phage display vectors, has the advantage of containing two long DNA "stuffer" fragments for easier differentiation of the correctly cut forms of the vector. Moreover, the fragment at the heavy-chain cloning site contains an acid phosphatase-encoding gene allowing an easy distinction of the *Escherichia coli* cells containing the heavy-chain fragment coding cDNA (Burioni *et al.*, 1997). In pCM transcription of heavy-chain Fd/*gene III* and light chain is driven by a single *lac*Z promoter and the heavy-chain Fd/coat protein III and the light chain are directed to the periplasm by the *pelB* and *ompA* signal peptide respectively. These features contribute to the molecular stability of the vector (Barbas C.F., 2001).

pCM was used to generate a human combinatorial phage display antibody library. Biopanning of the combinatorial phage display antibody library against the recombinant NP of Influenza A virus H1N1 (A/Puerto Rico/8/34/Mount Sinai) allowed the selection of specific human recombinant monoclonal antibodies. The selected antibody showed a specific reactivity in immunofluorescence assay against influenza-infected cells. This antibody can find potential applications for the setting of a diagnostic procedure for detection of Influenza A virus infection.

MATERIAL AND METHODS

Vector construction

The vector pCM is characterized by two long DNA "stuffer" fragments. The fragment at the heavy-chain cloning site contains an acid phosphatase-encoding gene (Burioni *et al.*, 1995). In pCM transcription of heavy-chain Fd/*gene III* and light chain is driven by a single *lac*Z promoter and the heavy-chain Fd/coat protein III and the light chain are directed to the periplasm by the *pelB* and *ompA* signal peptide respectively. A complete description of the construction of pCM is reported on the on-line supplementary material.

Lymphocyte RNA preparation and library construction

Total RNA was prepared from 5 mL of bone marrow of a 63-year-old man whose serum resulted positive for the presence of anti-NP antibodies in ELISA. Construction of the IgGl/k Fab library using the pCM surface display system was performed as described (Plaisant *et al.*, 1997).

Panning of the combinatorial library to select antigen binders

Panning of the combinatorial library was carried out as described (Plaisant *et al.*, 1997; Williamson *et al.*, 1993) using recombinant NP of Influenza A virus H1N1 (A/Puerto Rico/8/34/Mount Sinai) (IMGENEX)-coated ELISA plates (Costar). Phage (0.1 mL/well) at a concentration of 10^{12} phages/mL were used for each round of panning, and the number of eluted phages was determined (Plaisant *et al.*, 1997; Zebedee *et al.*, 1992). Phages from the final round of panning were used for the production of soluble Fab-expressing phagemid system as previously described (Bender *et al.*, 1993). *Escherichia coli* XL-l-Blue (Stratagene) strain transformed with Fab expression vector was used as a source of Fab molecules for further characterization.

Preparation and ELISA screening of soluble fab fragments

Fabs were prepared as bacterial supernatants through a freeze-thawing procedure as reported (Barbas *et al.*, 1992; Burioni *et al.*, 1998b). To assess specificity, supernatants were screened in an ELISA format (Plaisant *et al.*, 1997) with equivalent amounts of the antigen against which they

were initially panned and with bovine serum albumin (Sigma) used as a control preparation. Clones yielding an OD_{450}>0.8 in ELISA against recombinant NP (IMGENEX) were considered positive and further characterized.

Nucleic acid sequencing

The nucleic acid from ELISA-positive clones was obtain by Spin Miniprep Kit (Qiagen) and sequencing was performed on a 373A automated DNA sequencer (Perkin-Elmer).

Primers for the elucidation of heavy-chain sequence were: SEQGb (5'-GTCGTTGACCAGGCA GCCCAG-3') hybridizing to the (+) strand. For the light chain, SEQKb primer (5'-ATAGAAGTT GTTCAGCAGGCA-3') were used binding to the (+) strand.

Immunofluorescence of virally infected cells

Antibody Fabs found to be reactive with Influenza A NP (IMGENEX, San Diego, CA, USA) following ELISA screening were used in immunofluorescence studies using Madin-Darby Canine Kidney Cells (MDCK) infected with Influenza A/Puerto Rico/8/1934 virus (ATCC n. VR-1469) as previously described (Burioni *et al.*, 2010). As positive control was used the commercially available anti-hemagglutinin (HA) C179 antibody (Takara). As negative control was used the human anti-HCV/NS3 antibody (Chandra *et al.*, 2010).

RESULTS

A human combinatorial phage display IgGl/k antibody library with an estimated size of $2x10^7$ members was constructed using the novel vector pCM (Figure 1). Phages from the library were affinity selected against the recombinant Influenza A NP (A/Puerto Rico/8/34/Mount Sinai). Enrichment of antigen-specific phages, as determined by the number of phages eluted from NP-coated ELISA wells, was measured through 4 rounds of library panning. About 15-fold amplification was observed

TABLE 1 - *Number of phages eluted over four rounds of panning, challenging the antibody repertoire library constructed from and anti-Influenza A/NP-positive patients against Influenza A NP-specific antigen and against a control antigen (BSA).*

Round of panning	*Influenza A-NP antigen-coated wells*	*Control antigen (BSA)-coated wells*
1st	$1.7x10^6$	$1.4x10^6$
2nd	$6x10^6$	$2x10^6$
3rd	$8.5x10^6$	$3x10^6$
4th	$3x10^7$	$2x10^6$

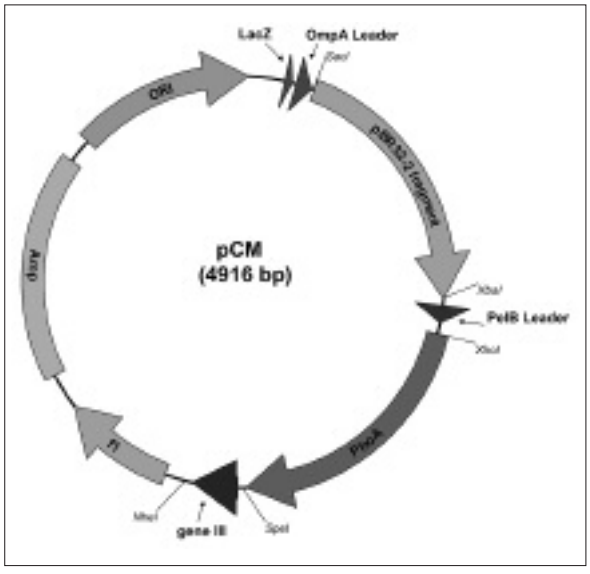

FIGURE 1 - *Schematic representation of pCM vector.*

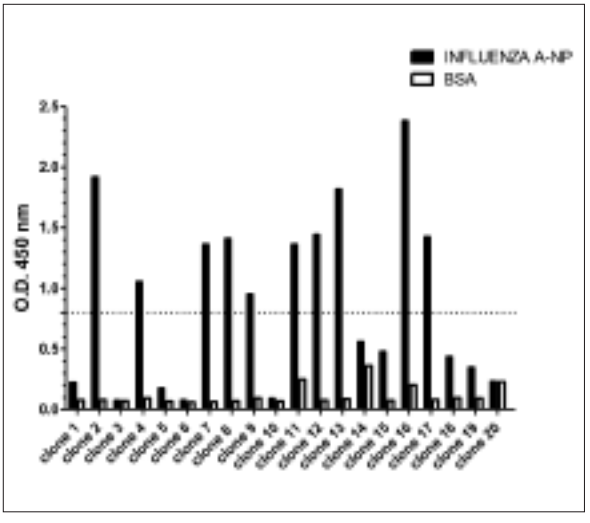

FIGURE 2 - *Results of ELISA performed with soluble Fabs obtained from the final round of panning against Influenza A NP antigen and BSA (negative control).*

TABLE 2 - *Amino acidic sequence of heavy-chain variable of the described anti-Influenza A/NP- specific antibody.*

FR1	*CDR1*	*FR2*	*CDR2*	*FR3*	*CDR3*	*FR4*
LESGPRLL KPSETLSL SCSVS	GGSISSYF	WTWIRQPP GKGLEWIGS	IYYTGST	NYSPSLTSRVTISAD SSKNHFSLKLTSVT AADTAVYYC	ARDDAQFPR VLQY	WGQGAL VTVSS

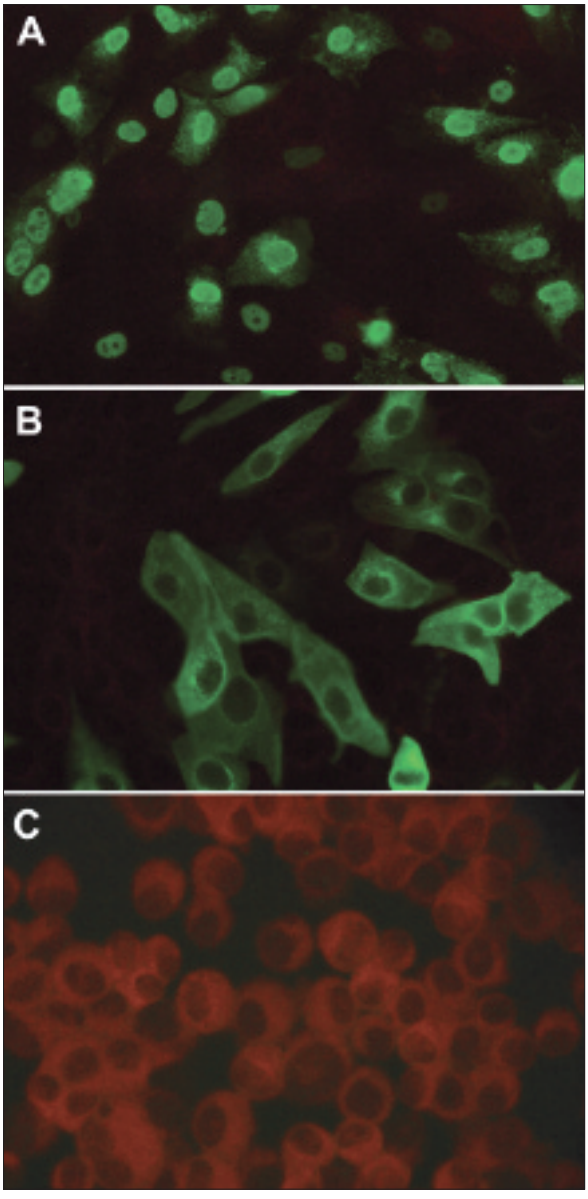

FIGURE 3 - *Immunofluorescence on A/Puerto Rico/8/1934-infected MDCK cells stained after 8 hours of infection with human monoclonal Ab NP1 (panel A), mouse anti-HA C179 antibody (B), negative control anti-HCV/NS3 antibody (C).*

(Table 1). When soluble Fabs were analyzed against the Influenza A NP-specific antigen, 10 out of 20 clones obtained from the final round of panning gave positive results in ELISA (Figure 2). DNA sequences of the heavy-chain CDR3 (Data not shown) variable domains were determined for all the Influenza A NP-specific clones and revealed that the selected clones share the same sequence (Table 2). This clone was named NP1.
NP1 Fab was tested in immunofluorescence assay against MDCK cells infected with Influenza A/Puerto Rico/8/1934 virus. As shown in figure 3 panel A NP1 antibody featured a clear and strong nuclear pattern against A/Puerto Rico/8/1934-infected cells. No reactivity was demonstrated when was used the anti-HCV/NS3 control antibody (Figure 3 panel C).

DISCUSSION

Combinatorial antibody libraries displayed on the surface of phages generated from human sources of B lymphocytes have been used for the isolation of antibodies against a wide range of viral pathogens. The successful molecular cloning of the antibody repertoire importantly depends from the phagemidic vector utilized for the construction of the library. In this work we describe a novel phagemidic vector, named pCM, characterized by two long DNA "stuffer" fragments. The fragment at the heavy-chain cloning site contains an acid phosphatase-encoding gene allowing an easy distinction of the *Escherichia coli* cells containing the heavy-chain fragment coding cDNA. In pCM transcription of heavy-chain Fd/*gene III* and light chain is driven by a single *lac*Z promoter and the heavy-chain Fd/coat protein III and the light chain are directed to the periplasm by the *pelB* and *ompA* signal peptide respectively.
pCM was utilized to generate a human combinatorial phage display IgGl/k antibody library with

a size of $2x10^7$ members. The biopanning selection of the library carried out against the recombinant NP of Influenza A virus H1N1 (A/Puerto Rico/8/34/Mount Sinai) allowed the isolation of a monoclonal antibody specific for the NP antigen, named NP1. When tested in immunofluorescence assay against MDCK cells infected with Influenza A/Puerto Rico/8/1934 virus the antibody showed a very clear and strong nuclear signal underling its high specificity.

Considering the high level of conservation of the NP among all influenza A strains (Portela *et al.*, 2002; Shu *et al.*, 1993), antibodies specifically recognizing this protein could find potential application for the diagnosis of the hypervariable Influenza A virus infection.

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