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Eight novel single chain antibody fragments recognising VP2 of foot-and-mouth disease virus serotypes A, O, and SAT 2

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ABSTRACT

Foot-and-mouth disease virus (FMDV) exhibits a high degree of antigenic diversity among its serotypes, requiring several anti-FMDV antibodies for its laboratory diagnosis, which complicated the used techniques. To conquer this cumbersome, we developed a new panel of different single-chain fragment variable (scFv) for serotype-independent detection of FMDV. The recombinant VP2 capsid protein, as a relatively conserved protein among FMDV serotypes, was expressed in *E. Coli*, and injected in mice. Spleen's RNA was extracted for isolating the coding sequences of IgG variable domains that were assembled into repertoires of scFv. Phage library displaying scFv was constructed with $\sim 1.9 \times 10^8$ plaque forming units. Characterization of the library showed eight of unique scFvs, which were expressed as bacterial periplasmic proteins with apparent molecular weight of ~27 kDa. Our data revealed the broad-spectrum binding affinity of the eight scFvs as both coating and tracing antibodies to FMDV serotypes A, O, and SAT 2.

1. Introduction

Foot-and-mouth disease (FMD) is an important contagious viral disease of all domestic and wild cloven-hoofed animals worldwide (Knight-Jones and Rushton, 2013). The disease remains enzootic in many countries, like Egypt, demanding effective vaccination programs, accurate diagnostic and serosurveillance tools in addition to solid control measures to abolish virus spread within a population (Reid et al., 2001; Jamal and Belsham, 2013).

Foot-and-mouth disease virus (FMDV), the causal agent of FMD, occurs as seven antigenically distinct serotypes (SAT 1, SAT 2, SAT 3, O, A, C and Asia 1) (Sumption et al., 2008), and has a single-stranded RNA molecule surrounded by a capsid composed of four structural proteins VP4, VP2, VP3 and VP1 (Jackson et al., 2003). Only, three of them (VP2, VP3, and VP1) are responsible for shaping the virus antigenicity due to the natural barring of VP4 from the viral-surface (Logan et al., 1993). VP4 is the highly conserved structural protein, VP1 is the most variable, VP2 and VP3 are relatively conserved, among FMDV serotypes (Jackson et al., 2003). VP2 antigenic sites have been determined (Marquardt et al., 2000; Mateu et al., 1990), and highly conserved linear epitopes exist on the VP2 protein (Freiberg et al., 2001; Yang et al., 2007). Furthermore, VP2 has been shown to play a role in FMDV antigenicity and immunogenicity, as substitutions in its amino acid sequence generated antigenic diversity and leading to changes in pathogenicity and replication properties (Xue et al., 2012).

FMDV exhibits a high degree of antigenic divergence among the seven serotypes, due to the error-prone transcription of the RNA-dependent RNA polymerase (Bachrach, 1968, Domingo et al., 2003), resulting in lack of cross-reactivity. Accordingly, specific antibody to each serotype should be used for consistent laboratory diagnosis of FMDV, thus anti-FMDV antibody to each of the seven serotypes along with sophisticated techniques are required for comprehensive FMDV laboratory diagnosis (Rodriguez and Gay, 2011). Furthermore, these antigenic variations are the major hurdles to the development of an assay for the serotype-independent diagnosis of FMDV (Yu et al., 2011). Development of such an assay can be used to assist primary field diagnosis of clinically suspected animals that would speed up the disease control (Muller et al., 2008).

Monoclonal antibody (MAb), are effectual molecules as either diagnostic or therapeutic tools. Use of MAb increases the accuracy and specificity of laboratory diagnosis in comparison to polyclonal antisera (Couture and Heath, 1995). Production of hybridoma-derived MAb is laborious and suffers from instability and storage troubles, resulting in reduction or loss of their activity. Recently, recombinant MAb (rMAb), have been developed as a versatile alternative to the hybridoma-derived ones (Yang et al., 2007; Ferris et al., 2009).

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Single-chain variable fragment (scFv) is one of the rMAb types that can be produced on a large scale by gene expression in *E. coli* (Reid et al., 2001; Ferris et al., 2010). ScFv is the smallest structure form of antibody which is still able to keep the whole antibody function of binding with an antigen. It is composed of a variable light chain linked to a variable heavy through a flexible sequence of polypeptide. ScFv is considered a prevalent rMAb because it can be easily bioengineered into multivalent, larger and conjugated forms suitable for diverse clinical applications as well as in the diagnostic realm (Baxt and Becker, 1990; Jackson et al., 2000).

Phage display technique has been invented in 1980s with uprising of novel methods for discovery and development of rMAb (Barbas et al., 1991). It provided a robust technology for generating *in vitro* peptide affinity through mimicking the immune system amplification and selection strategies (Cwirla et al., 1990).

This study was designed to produce VP2 specific scFv recombinant antibodies, using phage display technique, to provide a rapid, simple, and serotype-independent diagnostic tool. That could be a substitute for several sets of serotype-specific anti-FMDV antibody required in the currently used ELISAs. Besides, it might be of supporting value for primary FMDV diagnosis in suspected animals and for appropriate control procedure.

2. Materials and methods

2.1. Antigen preparation

The nucleotide sequence of FMDV VP2 was retrieved from an Egyptian SAT 2 isolate (gb | AAZ83686) and perceptively synthesized to be directionally inserted into the $P^{Gex-4t1}$ expression vector (GE Healthcare, Life Sciences, Logan, UT, USA) in-frame with the glutathione S-transferase (GST) encoding gene. The recombinant $P^{Gex-4t1-VP2}$ was transformed into *E. coli* BL21 and protein expression was verified using 15% SDS-PAGE analysis according to standard procedure (Laemmli, 1970). The expressed VP2- GST was purified via Glutathione resin (GE Healthcare, Life Sciences, Logan, UT, USA). Recombinant VP2 (rVP2) was liberated from GST-fusion protein by *Thrombin* enzyme and eluted from Sepharose beads, as instructed by the manufacturer.

2.2. Mice immunization

Two female BALB/c mice 21days old obtained from Theodor Bilharz Research Institute, Egypt, were treated in accordance with the principles and policies of the National Institute of Health (NIH) animal care. Mice were injected with the rVP2 upon the approval of research ethics committee, in National Center for Radiation Research and Technology (REC-NCRRT/13A/18); following the injection schedule presented in Table 1. The primary immune response was initiated by intraperitoneal injection of mice with a 50 µg of the rVP2 emulsified in complete Freund's adjuvant, followed by four subsequent intravenous boosters, at weekly interval, each with a 100 µg of rVP2 emulsified in incomplete Freund's adjuvant that was excluded from the last booster. Seven days post the fifth injection, sera were collected for assessment of anti-rVP2 seroconversion; then mice were euthanized and spleens were gathered for RNA isolation.

Table 1
Mice injection schedule and antigen doses.

Injection	Antigen-dose	Route	Freund's Adjuvant	Days of sera collection post the 1st injection
1	50 µg	Intraperitoneal	Complete	0
2	100 µg	Intravenous	incomplete	7
3	100 µg	intravenous	incomplete	14
4	100 µg	Intravenous	incomplete	21
5	100 µg	intravenous	Without adjuvant	28

2.3. Mice humoral response to rVP2

The humoral immune response represented by the elicited anti-rVP2 Ab in the collected sera from immunized mice on days as presented in Table 1were assessed by indirect ELISA. Briefly, a 96-well microplate (Nunc, Denmark), was coated with 100 ng/well of rVP2 protein diluted in carbonate and bicarbonate buffer pH 9.6 and kept at 4 °C for overnight. Wells were washed with Phosphate-Buffered Saline containing 0.05%Tween 20 (PBST), then a 100 µl/well of 3% bovine serum albumin (BSA) was added and the microplate was incubated at 37 °C for 2 h. Wells were then washed twice with PBST; a 100 µl/well of each serum sample (diluted 1:1000 in PBS) was added in duplicates and allowed to react for 2 h at 37 °C. The collected serum on 0 day prior to first immunization was used as a negative control. Wells were intensively-washed with PBST and a 100 µl/well of rabbit anti-mouse IgG (Sigma Aldrich) conjugated with Alkaline phosphatase (AP) was added at 1:20000 dilution and re-incubated for 1 h. The excess and unbound conjugated antibody was removed by washing with PBST and the reaction was developed by adding 100µl/well of p-Nitrophenyl Phosphate substrate solution. After incubation at 37 °C for 30 min, 3 M NaOH was added to stop the reaction and absorbance was determined at 450 nm using microplate reader (iMark, Bio-Rad).

2.4. ScFv cloning and library construction

The strategy used for cloning and assembly of variable heavy (V_H) and variable light (V_L) coding fragments was scheme diagramed in Fig. 1. Briefly, total RNA was extracted from dissected spleens from rVP2 immunized mice using SV-total RNA isolation system (Promega). The protocol of RevertAid Reverse Transcriptase kit, Thermo Scientific, was used for generating the first-strand complementary DNA (cDNA). The IgG variable domains (V_H and V_L) were amplified through two subsequent PCRs using three pairs of primers designed according to the V-BASE database of IgG variable domains germline. In the first PCR, repertoires of V_H and V_L coding sequences were amplified separately from cDNA, under the following PCR conditions: 94 °C for 3 min followed by 30 cycles of 94 °C for 35 s, 58 °C for 35 s, and 72 °C for 45 s.

Equal amounts of amplified V_L and V_H repertoires (~100 ng/each) were combined to be assembled through the second PCR called splicing overlapping extension (SOE)-PCR (SOE-PCR). PCR was started by 94 °C for 3 min followed by five cycles of 94 °C for 35 s, 60 °C for 35 s, and 72 °C for 1 min, five cycles of 94 °C for 35 s, 72 °C for 45 s, and 72 °C for 1 min and thirteen cycles of 94 °C for 45 s, 68 °C for 1 min, and 72 °C for 1 min.

Assembled scFv repertoires were digested with *Sfi*I and $P^{ADL-22c}$ phagemid (Antibody Design Labs, San Diego, USA) was digested with *Bgl*I (Thermo Fisher Scientific, USA) which generated the identical overhangs (GCCNNNN/NGGC), as the sites opened with *Bgl*I were re-ligated with that opened with *Sfi*I. Digested scFv repertoires were batch-ligated into $P^{ADL-22c}$ phagemid using T4 DNA ligase (New England Biolabs, UK), in fifteen ligation reactions transformed separately into *E. coli* TG1 competent cells. Afterwards, all transformations were mixed and an aliquot was taken and serially-diluted 10^3, 10^4, and 10^5-fold. Dilutions were plated overnight at 37 °C on Luria-Bertani (LB) agar plates (containing 100 µg/ml ampicillin and 100 mM glucose), to be used later for verifying the library size and quality. The rest of transformation mixture was plated onto larger LB plates under the same conditions. The resulting bacterial clones were scrapped together into LB broth medium. Cells were collected by centrifugation and re-suspended in 2xYT broth medium containing 15% glycerol and kept at –80 °C as a library stock.

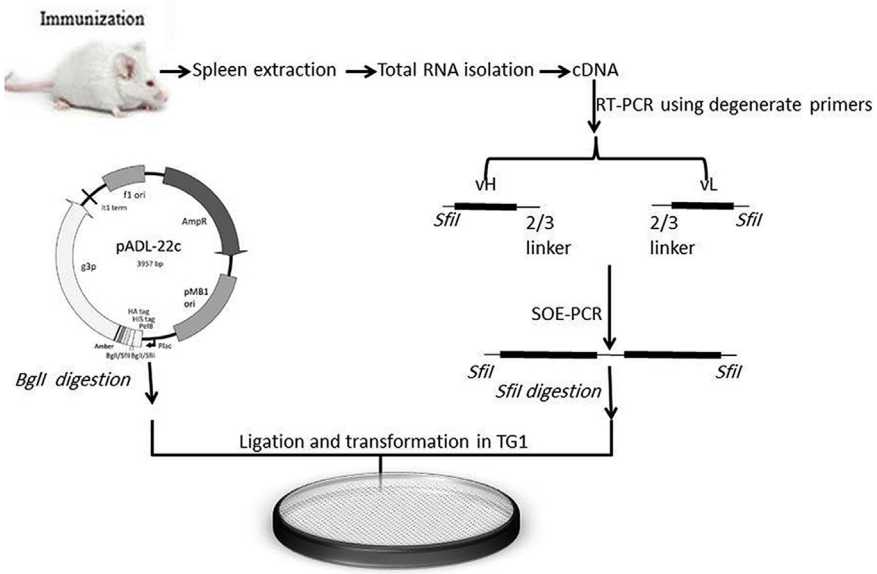

Fig. 1. Schematic diagram showing the construction and assembly of scFv from rVP2- immunized mouse in addition to the cloning strategy in $P^{ADL-22c}$ phagemid.

2.5. Rescue phage and bio-panning

An aliquot of the library stock was activated by inoculation into 2xYT medium containing ampicillin (100 µg/ml) and 1% glucose, at 37 °C for 3 h with shaking. Grown culture was infected with approximately 10^{11} plaque forming units (pfu) of M13K107 helper phage (Antibody Design Labs, San Diego, USA), and left for 30 min at room temperature (RT) without shaking, then was harvested by centrifugation and resuspended in a larger volume of 2xYT followed by overnight shaking at 37 °C. After centrifugation, the rescued phages were precipitated from the supernatant by adding PEG/NaCl solution (2.5 M PEG and 20% NaCl). Pellet containing phages resuspended in 1x PBS and was clarified from bacterial cells, cell debris and phage aggregates by centrifugation. Absorbance of solution containing phage (50-fold dilution) was measured at OD_{260}.

Purified phages were exposed to three rounds of panning against the rVP2. Briefly, an ELISA microplate was coated with rVP2 (5 µg rVP2/well), at 4 °C for overnight, after washing and blocking, rescued phage was added to the coated wells for 1 h. Unbound residual phages were discarded; after ten times washing with PBST, phages were eluted with 100 µl of 100 mM freshly prepared tri-ethylamine (Sigma-Aldrich). Eluted phages were neutralized by 1 M Tris pH 7.4 and amplified by immediately infection of exponentially grown *E. coli* TG1 (Antibody Design Labs, San Diego, USA, Cat.# PC001). Rescued phages were harvested and subjected to other two cycles of subsequent panning for enriching rescued phages displaying specific scFv.

For thorough selection, reduced rVP2 antigen concentrations (5, 3, and 2 µg) and increased washing times (10, 15, and 20 times), were applied, for subsequent panning rounds. Input-phages through all rounds of panning were maintained at approximately 10^{11} pfu. As a negative control, a duplicate well free from coating antigen was considered during all panning rounds.

The pfu of each input and output (eluted) phage were estimated through the three panning rounds. Difference between input and output phage titers of each panning round was used for calculation of the enriching factor.

2.6. Phage polyclonal ELISA

Polyclonal phage ELISA was applied on the phage populations obtained after each round of panning to see whether they have been enriched for rVP2-specific phages. 96-well ELISA microplates were coated with 100 ng/well of rVP2 protein in a number of wells equal to the number of panning rounds. The microplates were incubated at 4 °C for overnight, washed and blocked using 5% skimmed milk (in PBS pH 7.6) for 2 h at RT. The microplates were washed and phages were added (10^{11} pfu phages in final volume of 100 µl); this was performed for phages from before and after each round of panning. After washing with PBS/0.05%Tween-20, 100 µl/well, a mouse anti-M13 P^{III} monoclonal antibody (MAb; Antibody Design Labs, San Diego, USA) diluted 1:6000 was added and incubated at 37 °C for 2 h. After subsequent washing, a 100 µl/well of alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG (Sigma Aldrich; 1:20000) was added. Following 1 h incubation at 37 °C, the microplates were washed and detection was developed by adding 100 µl/well of p-Nitrophenyl Phosphate substrate solution. After incubation at 37 °C for 30 min, reaction was stopped by 3 M NaOH. The microplate reader (iMark, Bio-Rad) was used to measure the absorbance at 450 nm.

2.7. ScFv-phage specific (monoclonal) ELISA

Random of individual colonies from after each panning were picked up and cultured into deep 96-well microplates containing 2xYT medium with 1% glucose and 100 µg/ml ampicillin (a copy of the same colony was plated on LB agar plate and kept as reference). Phage rescue was performed by adding ~10^{11} pfu of M13KO7 to the cultures, followed by shaking at 37 °C for 2 h at 150 rpm then, centrifugation at 1000 × g for 15 min. The pellet was resuspended in 400 µl of LB broth medium and grown at 30 °C overnight with shaking at 250 rpm. After centrifugation, the phage-containing supernatant was screened by ELISA as described above in section 2.6. Except that anti-His Ab tag (Intron, Beijing, China) was used as the first antibody instead of anti-P^{III}.

2.8. Molecular characterization of scFv

The recombinant $P^{ADL-22c\text{-}vp2}$ phagemid was extracted from positive clones that were identified by monoclonal ELISA and subjected for sequence analysis. The generated scFv sequences were blast searched on Immunoglobulin BLAST (IgBlast, https://www.ncbi.nlm.nih.gov/igblast/), GenBank database and their relatedness to other scFv from mice was measured using an integrative database of germ-line variable genes (VBASE2, http://www.vbase2.org/). Furthermore, scFv generated sequences were aligned together using MegaAlign lasergene software 4.0, and complementarity-determining regions (CDRs) and frame

readings (FRs) were identified according to the Kabat numbering (Kabat, 1991).

2.9. ScFv expression and purification

To express scFv free of P^{III} protein, an amber codon non-suppressive *E. coli* strain (SS320) was used for expression. The $P^{ADL-22c}$ phagemids harboring the unique scFvs coding sequence were separately introduced into competent SS320 and expression was induced by IPTG. Since $P^{ADL-22c}$ phagemid has the PelB leader sequence for exporting scFv expression in the bacteria periplasmic space, total periplasmic proteins were extracted by centrifugation of overnight grown culture then, the pellet was resuspended in cold TES buffer (0.2 M Tris-HCl (pH 8), 0.5 mM EDTA, 0.5 M sucrose). After incubation for 1 h on ice, proteins were collected by centrifugation at 4000 rpm for 25 min and re-suspended in TES buffer. The extracted Periplasmic proteins were separated on SDS-PAGE 15% and were analyzed by Western blot. Briefly, proteins were transferred onto a nitrocellulose membrane, followed by blocking with 4% BSA at 4 °C for overnight. After washing with TBS containing 0.1% Tween 20, mouse anti-His antibody (Intron, Beijing, China) was diluted 1:6000 in TBS and added for 2 h with gentle shaking at RT. After washing, goat anti-mouse conjugated with Alkaline phosphatase (Sigma, USA; diluted 1:20000) was added as a secondary antibody and incubated for 1 h at RT. The membrane was thoroughly washed and the color reaction was developed with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP), solution. The total periplasmic proteins from SS320 that has been transformed with non-recombinant $P^{ADL-22c}$ were loaded as a negative control.

After expression analysis by western blotting, recombinant scFv protein was purified from the selected clones. Briefly, a 100 ml of overnight culture from each clone was centrifuged, and the total periplasmic proteins were extracted as described above. ScFv peptide with C-terminal 6x His-tag was purified from resuspended pellets using Ni-NTA agarose resin (Qiagen, Germany) as was described in the manufacturer's instructions. Eluted scFv containing imidazole was subjected to dialysis against 1x PBS for overnight at 4 °C.

2.10. ScFv-rVP2 binding affinity

The scFv binding affinity for rVP2 was validated by indirect-ELISA. Briefly, a 96-well ELISA microplate was coated with 0.04 µg rVP2 protein/well (diluted in coating buffer pH 9.6) and kept for overnight at 4 °C. As a negative control, BSA was used as a coating antigen in parallel. Wells were washed and residual free spaces were blocked with BSA. Following washing, scFv diluted in PBS containing 3% BSA was added for 2 h at RT. After washing, mouse anti-His labeled with alkaline phosphatase (Thermo Fisher Scientific, USA; diluted 1:6000) was added for 1 h at RT, followed by washing with PBST. The reaction was developed by 100 µl/well of p-Nitrophenyl Phosphate substrate solution, and after incubation at 37 °C for 30 min it was stopped by 3 M NaOH. Absorbance at 450 nm was measured using Microplate reader (iMark, Bio-Rad).

2.11. FMDV serotype-independent detection using developed scFv

2.11.1. Viruses, cells, cultures and antibodies

Egyptian local isolates of FMDV serotypes O_1/Manisa, A/Egy/2006, and SAT 2/Egy/2012, were propagated in an appropriate bio-containment level at the quality control laboratory (QCL), Veterinary Sera and Vaccines Research Institute (VSVRI), Abbassia, Cairo, Egypt. BHK_{21} clone 13 cells from baby hamster kidney were used and maintained in minimum essential medium with Eagle's salts (MEME) supplemented with heat-inactivated 5–10% newborn calf sera (NCS), 100 U/mL penicillin, 100 µg/ml streptomycin, and 25 i.u./mL mycostatin. Guinea pig antisera against FMDV O/Manisa (Cat.#DDT-FMDV-O-GP), A22 (Cat.# DDT-FMDV-A-GP) and SAT 2 (Cat.# DDT-FMDV-SAT 2-GP) were obtained from IAH, Pirbright, UK. Goat anti-guinea pig-HRP labeled IgG (PA1–28597) was obtained from Thermo Scientific.

2.11.2. Indirect ELISA

The scFv binding affinity as a tracing antibody to different FMDV serotypes (serotype-independent), was evaluated by indirect ELISA exactly as above described in section 2.10., except replacing the rVP2 as a coating antigen with FMDV serotypes O_1/Manisa, A/Egy/2006 and SAT 2/Egy/2012. Cell culture medium was used as a negative control coating antigen against the FMDV.

2.11.3. Indirect antibody sandwich ELISA

The scFv binding affinity as a coating antibody for FMDV was validated by indirect double antibody sandwich ELISA. Briefly, the recombinant scFv (1 mg/ml) diluted 1:1000 in coating buffer pH 9.6, was coated into a 96-well ELISA microplate (Nunc, IL, USA) for overnight at 4 °C. FMDV-free serum from a naive calf was used as a negative antibody control. Wells were washed, and residual free spaces were blocked with 3% BSA. Following washing, inactivated FMDV serotypes O_1/Manisa, A/Egy/2006 and SAT 2/Egy/2012, were separately diluted in PBS and added to the wells, and incubation was continued at RT for an additional 2 h. The serotype-specific guinea pig antiserum against each serotype was added to wells in PBS and reaction was allowed for 2 h at RT, followed by washing. The HRP conjugated goat anti-guinea pig IgG diluted 1:1000 in PBS containing 3% BSA was added. After incubation at 37 °C for 1 h, wells were washed, and 100 µl TMB-ELISA substrate (KPL, MD, USA) was added. After incubation at 37 °C for 30 min, reaction was stopped with 2 M sulfuric acid, and a microplate reader (Vmax kinetic) was used to read the plates at wavelength of 450 nm.

3. Results

3.1. Expression of VP2 in E. coli

The VP2 gene was definitely expressed in *E. coli*BL21as revealed by SDS–PAGE analysis (Fig. 2). The rVP2 fused to N-terminal of the GST had the expected molecular weight of approximately 48 KDa (22 KDa VP2 + 26 KDa GST). SDS-PAGE analysis showed a major purified band correspondent to the expected molecular weight of VP2 (~22 KDa) and only minor nonspecific contaminations were detected in the eluted fraction.

3.2. Immune response to recombinant VP2

The indirect rVP2-ELISA showed a significant increase in the anti-rVP2 Ab levels as represented by the OD_{405} values of tested sera from rVP2 immunized mice on 0, 7, 14, 21, and 28 days post immunization (Fig. 3). This result revealed competent binding activity of rVP2 as a coating ELISA antigen to anti-VP2 Ab in addition to its efficacy to induce a humoral immune response following a successful immunization protocol.

3.3. ScFv assembly and cloning

ScFv was constructed from cDNA template derived from the RNAs isolated from spleens of immunized mice. The integrity of extracted RNA was demonstrated by electrophoresis (not shown). V_H and V_L domains were correctly amplified by PCR at the expected molecular sizes of 400 and 360 bp, respectively (Fig. 4A). Repertoires of the two domains were joined together with a separating 45-mer-polynucleotide linker $(GGGGS)_3$ using a SOE-PCR, resulting in combinatorial repertoires of scFv with a molecular size of ~805 bp (Fig. 4B). ScFv fragments were inserted into $P^{ADL-22c}$ phagemid, between the PelB peptide leader sequence (MKYLLPTAAAGLLLLAAQPAMA) and the N-terminus of the phage M13 gene III (g3) sequence, for subsequent

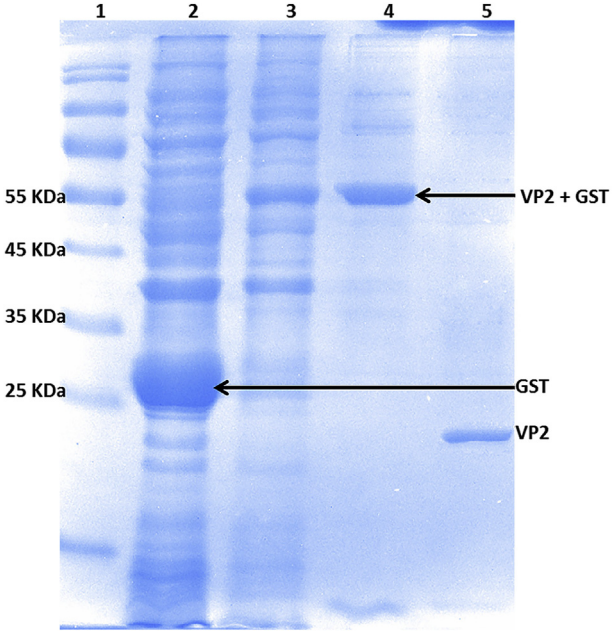

Fig. 2. SDS-PAGE shows the rVP2 protein. 1: protein marker; 2: cell-lysate of *E. coli* transformed with non-recombinant $P^{Gex-4t1}$ (arrow points to the GST protein); 3 and 4: show the rVP2 protein tagged with GST, in the cell-lysate of *E. coli* transformed with recombinant $P^{Gex-4t1-VP2}$, and after being purified with Sepharose 4B, respectively (the arrow points to the rVP2 fused to GST), 5: purified rVP2 after being liberated from GST by *thrombin*.

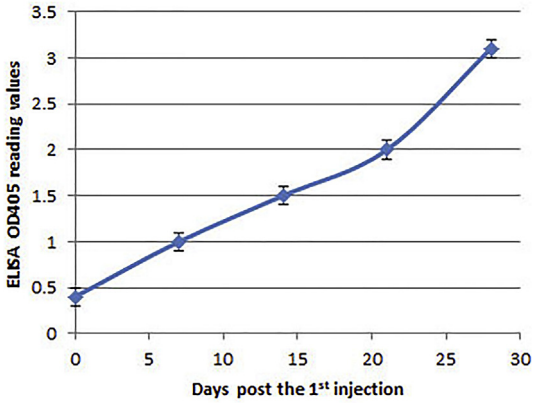

Fig. 3. Graphical representation of antibody response in mice against rVP2 after each immunization. High anti-rVP2 antibody levels were detected after 28 days.

driving of their secretion in the bacterial periplasmic space and displaying on phage, respectively. Since the PelB sequence was originally interrupted by a stop codon, the two last amino acid sequences MA were re-introduced through PCR for proper frame proofreading and cleavage. His and HA tags that were trailed by an amber codon were located between scFv and g3 sequences. Integration of scFv into $P^{ADL-22c}$ was confirmed by *Sfi*I digestion, where scFv correspondent band at the size of ~805 bp was released from the $P^{ADL-22c}$ whose size was ~4000 bp (Fig. 4C).

3.4. Library construction

ScFv phage library against rVP2 was constructed by transformation of the recombinant phagemids ($P^{ADL-22c\text{-}scFv}$) into *E. Coli* TG1, suppressing the amber codon located between scFv and g3 coding sequences that allowed the display of scFv antibodies on the N-terminus of g3 protein (P^{III}) of phage M13. A robust enriching factor was noticed

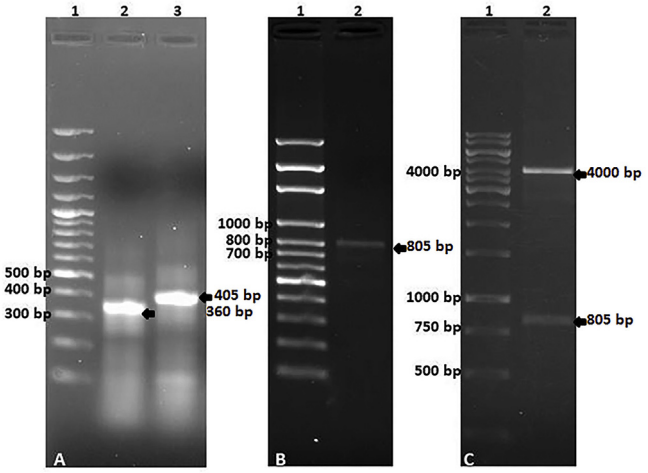

Fig. 4. Amplification of V_H and V_L coding sequences from cDNA prepared from rVP2- immunized mice and assembly of scFv. **(A)** PCR amplification of V_H and V_L repertoires. Lanes: (1) 100 bp DNA marker, (2 and 3): amplified repertoires of V_L ~ 360 bp and V_H ~ 405bp, respectively. **(B)** Assembly of amplified V_L and V_H repertoires and formation of scFv using SOE-PCR. Lanes: (1) 100 bp DNA marker, (2) Assembled scFv with linker (~805 bp). **(C)** Verification of scFv cloning in $P^{ADL-22c}$ phagemid by *Sfi*I digestion. Lanes: (1) 100 bp DNA marker, (2) released scFv insert (~805 bp) from the *Sfi*I cut $P^{ADL-22c}$ phagemid (~4000 bp).

after library-infection with M13KO7 helper phage. Library-size was determined based on the number of grown colonies (the dilution factor was considered) as a total number of 1.9×10^8 colonies from the cultured library was exposed. Library recombination frequency of 100% was indicated through PCR screening for the presence of scFv in 19 randomly selected clones, where specific scFv DNA fragments were amplified with unique sizes of approximately 900 bp from all tested clones (supplementary file 1).

Specificity determination of scFv by Phage polyclonal ELISA showed that 88% of the selected clones reacted positively with the rVP2, while none was reacted with the BSA control antigen. Furthermore, the Phage monoclonal ELISA proved that 50 out of 150 (33%) individual clones were definitely reactive to rVP2 as manifested by high ELISA OD values.

Phage recovery was doubled to approximately 50-times after the third panning compared to the first panning round (Table 2), demonstrating a highly enriching factor of VP2 antigen-specific scFv.

3.5. Characterization of scFv genes

The coding sequences of the variable domains for selected scFv antibodies were determined and compared showing that eight scFvs out of fifteen were unique. These scfv unique sequences were examined for the full length and correct V_H-linker-V_L structure. Comparing their amino acids sequences along with Protein Data Bank (PDB) revealed that their V_H- and V_L-domains belong to the variable regions of mouse Ig with identities of 91% and 85% to the Ig kappa chain and Ig heavy chain, respectively. Blasting results of scFv deduced amino acids

Table 2
Estimation of enriching factor after each panning based on the difference between Phage input and Phage output (recovery) expressed as pfu.

Panning round	Phage Input (pfu*)	Phage recovery Output (pfu)	Enriching factor**
First	1×10^{12}	2×10^5	2×10^{-7}
Second	4×10^{11}	5×10^6	1.25×10^{-5}
Third	9×10^{10}	3×10^7	0.33×10^{-4}

* pfu: plaque-forming unit.
** Enriching factor = output phages/input phages.

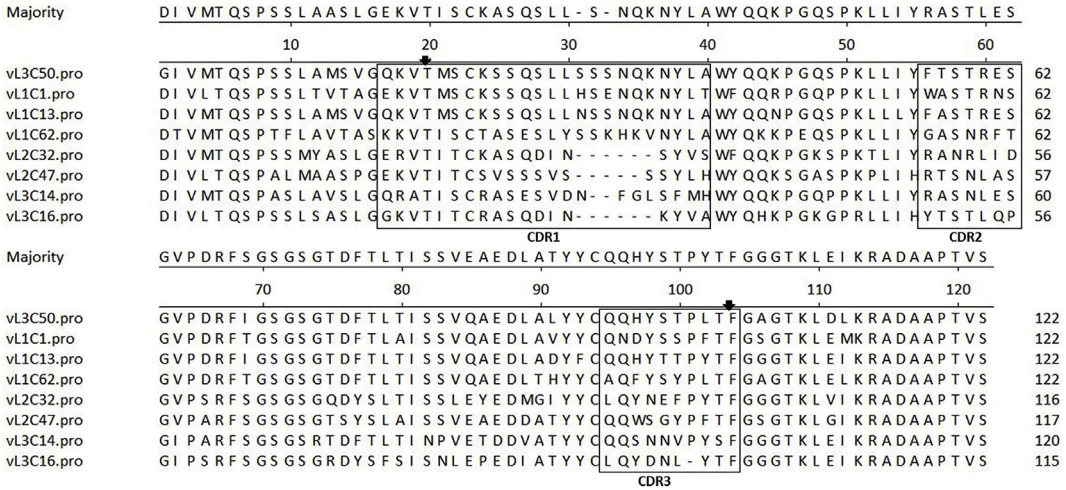

Fig. 5. Characterization the V_L amino acid sequences of unique scFvs. Using ClustralW, Lasergene software, three specific CDRs (boxed), separated by FRs were detected in V_L amino acid sequences. Arrows pointed to the conserved residues.

sequence, against the protein data bank showed a close relationship and specific hits with the Ig super family of the mouse antibody heavy chain and kappa chain (not shown). As well as it shows the coherent sequence of the produced scFvs to the other V libraries from the mouse IgG, they have the basic structure of scFvs, where the framework regions are highly conserved regions of variable portion of the antibody, while CDRs are hypervariable regions.

By multiple alignments of V_H and V_L deduced amino acid (aa) sequences and applying the Kabat numbering method (Kabat, 1991), six complementarity-determining regions (CDRs), three in each of the V_L and V_H domains, separated by four framework regions (FRs) were identified (Figs. 5 and 6). The recognized CDRs and FRs lengths and locations were presented in Table 3. Considerable conservation in the FRs, while conversely, significant variations in CDRs were observed. Furthermore, the CDR3 in V_H (HCDR3) was identified at 99–112 aa residues that exhibited a hypervariable sequence between 102 and 106 aa residues. The V_L and V_H sequences of the unique scFv clones (Mab3c50, Mab3c16, Mab3c14, Mab2c47, Mab1c32, Mab1c1, Mab1c1 and Mab1c62), were deposited in GenBank database (Table 4).

3.6. Expression and purification of scFv antibodies

Selected scFv clones were separately transformed into a non-suppressive amber codon *E. coli* SS32. After induction, periplasmic protein extracts were separated on SDS–PAGE. All extracts showed a distinct protein band at an apparent molecular weight of ~27 KDa correspondents to the expected size of recombinant scFv (Fig. 7A). As the expressed scFv proteins were tagged with 6 His amino acids, they were recognized with an anti-His antibody in Western blot analysis (Fig. 7B). Ni-NTA affinity resins, which have the ability to specifically bind to 6 His tag polypeptides were used to purify the recombinant scFv proteins (Fig. 7C). Approximately 3 mg of each scFv recombinant protein was purified from 1 L of *E. coli* culture.

3.7. Verification of the scFv detection activity

To confirm that the purified scFv are still as biologically active as keeping their binding affinity to the rVP2 protein, the later was used as coating antigen in an indirect-ELISA. Results showed that all of the eight scFv are still able to recognize and react with the rVP2 protein (Fig. 8).

3.8. ScFv suitability for FMDV serotype-independent detection

The potential capacity of the eight scFvs for binding with different FMDV serotypes was exposed by conducting indirect rVP2-ELISA and

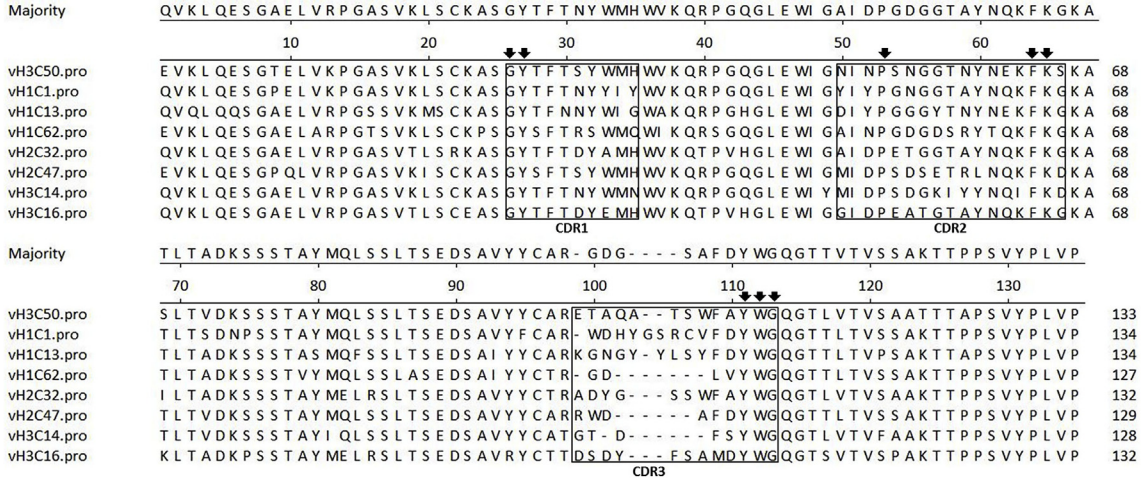

Fig. 6. Characterization the V_H amino acid sequences of unique scFvs. Using ClustralW, Lasergene software, three specific CDRs (boxed), separated by FRs were detected in V_H amino acid sequences. Arrows pointed to the conserved residues. High variable region in CDR3 was noticed.

Table 3
Identification of lengths and locations of CDRs and FRs in the generated scFvs, by applying the Kabat numbering method after multiple alignments of their V_H and V_L deduced amino acid sequences.

	FR1		CDR1		FR2		CDR2		FR3		CDR3		FR4	
	*Lo	**Le	Lo	Le	Lo	Le	Lo	Le	Lo	Le	Lo	Le	Lo	Le
V_H	1–25	25	26–35	10	36–49	14	50–66	17	67–98	32	99–113	15	114–135	24
V_L	1–23	23	24–40	17	41–55	15	56–62	7	63–94	32	95–103	9	104–22	19

*Lo (location) and **Le (length): are the location and number of amino acid residues in the sequence, respectively.

indirect double antibody sandwich ELISA. Nevertheless, the results of both ELISAs indicated their broad-spectrum binding affinity to A, O, and SAT 2 serotypes. Results of both ELISAs (Fig. 9), suggested suitability of the generated scFvs as FMDV serotype-independent detection tools.

4. Discussion

Diagnosis of FMDV is facing some difficulties due to absence of cross-reactivity between FMDV serotypes along with the considerable intratypic antigenic divergence as a result of FMDV quasispecies structure (Domingo et al., 1992).

In this study, a group of FMDV VP2 specific scFv recombinant monoclonal antibodies were generated, using phage display technique, to provide a rapid, simple, and serotype-independent diagnostic tool. In brief, a library of recombinant phage expressing and displaying scFv coding sequences was screened for specific binding to recombinant FMDV VP2 capsid protein (rVP2) by a technique called biopanning. Consequently, eight of those scFv were selected and validated for serotype-independent detection of various FMDV serotypes.

The conservation of VP2 sequence among the FMDV serotypes was earlier discussed (Jackson et al., 2003). Although, VP4 is the highly conserved structural protein among the FMDV serotypes, its natural barring from the viral-surface was evidenced (Logan et al., 1993). While, VP3 and VP2 are relatively conserved, and shared in FMDV shaping. VP2 was selected in this endeavor due to its essential role in FMDV antigenicity and immunogenicity, as substitutions in its amino acid generating antigenic diversity, and also lead to changes in FMDV pathogenicity and replication properties (Xue et al., 2012), in addition to the highly conserved linear epitopes subsistence which confirmed on the VP2 protein (Freiberg et al., 2001; Yang et al., 2007).

The obtained analytical data of VP2 deduced amino acid sequences from various FMDV serotypes confirmed that VP2 is a reasonably conserved protein (data not shown). Moreover, the FMDV rVP2 protein, being expressed in *E. coli* (Fig. 2), was found as immunogenic as it could induce a substantial specific antibody response in experimentally inoculated mice (Fig. 3). Although, earlier study showed that VP2 has no fixed position and therefore might has antigenic sites (Acharya et al., 1989), other investigations suggested weak antigenicity of VP2 (Curry

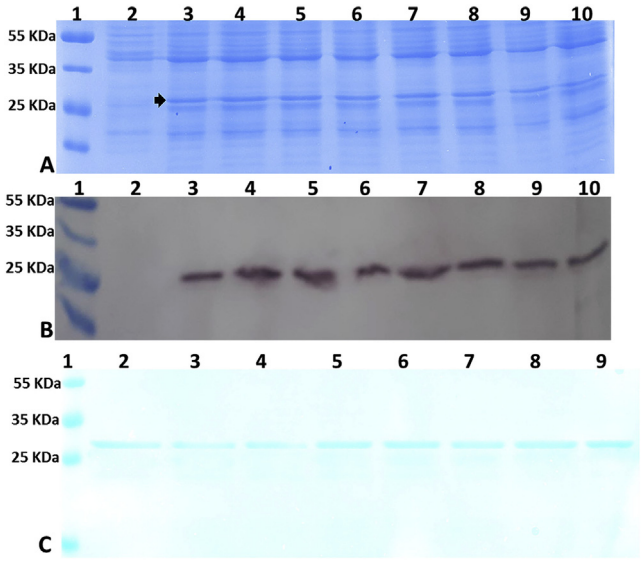

Fig. 7. Expression, verification, and purification of scFv-Abs. (A) 15%SDS-PAGE shows the expression of scFv-Abs in eight selected clones after three rounds of panning. Lane 1, protein marker; lane 2, SS320 transformed with non-recombinant P^{ADL22c}, lanes 3–10, SS320 transformed with recombinant $P^{ADL22c\text{-}scFv}$ from the clones Mab3c50, Mab3c16, Mab3c14, Mab2c47, Mab1c32, Mab1c1, Mab1c1 and Mab1c62, respectively. The arrow points to various scFv, molecular mass of ~27 kDa. (B) Western blot analysis to verify expression of scFv in *E. coli* using anti-His tag antibody. (C), Purification of scFv peptide with C-terminal 6x His-tag from the eight selected clones using the Ni-NTA agarose resin affinity chromatography.

Table 4
Informatics' ID, name and accession number of the V_L and V_H sequences of the unique scFv clones (Mab3c50, Mab3c16, Mab3c14, Mab2c47, Mab1c32, Mab1c1, Mab1c1 and Mab1c62), deposited in GenBank database.

Seq# ID	Seq# Name	Region	Accession	Length(bp)
BankIt2123387	Mab 3c50,SCFV,	heavy chain variable	MH476560	399
BankIt2123383	Mab 3c16,SCFV,	heavy chain variable	MH476559	396
BankIt2123376	Mab 3c14,SCFV,	heavy chain variable	MH476558	384
BankIt2123375	Mab 2c47,SCFV,	heavy chain variable	MH476557	387
BankIt2123373	Mab 1c32,SCFV,	heavy chain variable	MH476556	396
BankIt2123368	Mab 1c13,SCFV,	heavy chain variable	MH476555	402
BankIt2123364	Mab 1c1,SCFV,	heavy chain variable	MH476554	402
BankIt2123360	Mab 1c62,SCFV,	heavy chain variable	MH476552	381
BankIt2123193	Mab 3c50,SCFV,	light chain variable	MH476550	366
BankIt2123190	Mab 3c16,SCFV,	light chain variable	MH476549	345
BankIt2123188	Mab 3c14,SCFV,	light chain variable	MH476548	360
BankIt2123186	Mab 2c47,SCFV,	light chain variable	MH476547	351
BankIt2123185	Mab 2c32,SCFV,	light chain variable	MH476546	348
BankIt2123184	Mab 1c62,SCFV,	light chain variable	MH476545	366
BankIt2123182	Mab 1c13,SCFV,	light chain variable	MH476544	366
BankIt2123178	Mab 1c1,SCFV,	light chain variable	MH476543	366

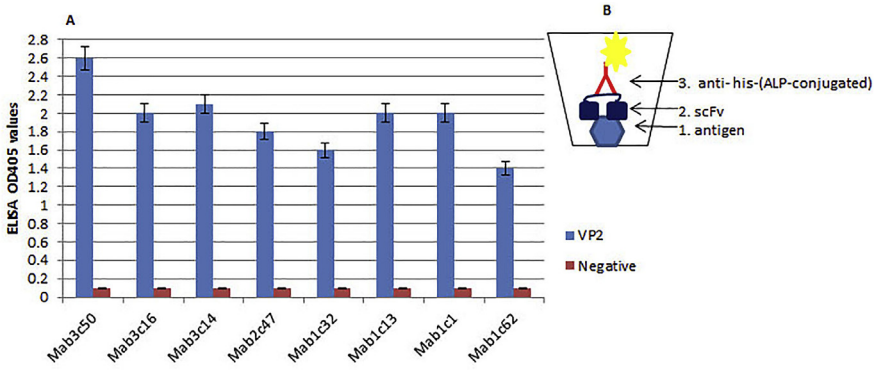

Fig. 8. Evaluation of structural stability of scFv-Abs after purification from *E. coli* using indirect antigen trapped ELISA. (A) An illustration showing ELISA assembly. Numbers (1, 2, and 3) represent the order of added components. (B) Partially purified scFv polypeptides were tested for its capability to interact with, and detect, rVP2. BSA was used as a negative control (red).

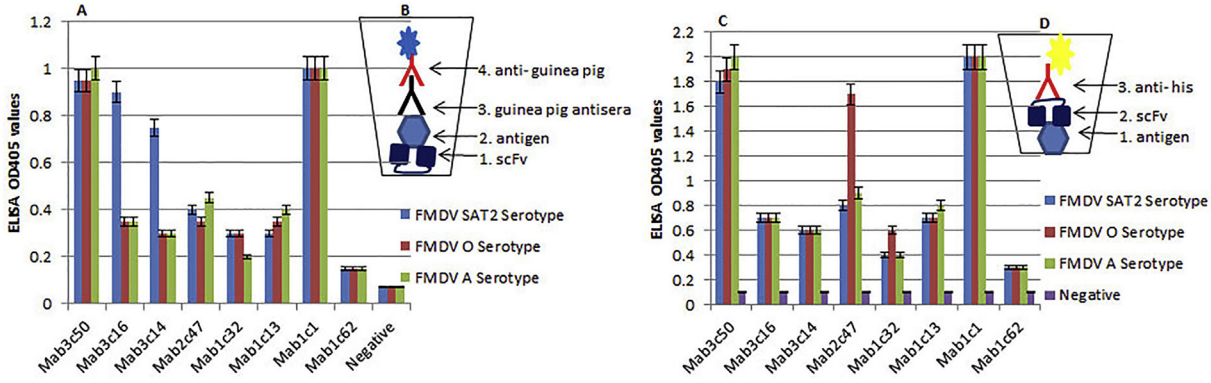

Fig. 9. Evaluation of using generated scFvs for FMDV serotype-independent detection. ScFvs were evaluated as capturing antibodies by Indirect sandwich ELISA (A), where serum from a naive calf FMDV-free was used as a negative control antibody. In B, the scFvs were evaluated indirect antigen coating ELISA, where BHK21 culture medium was coated as a negative control. B and D, diagrammed the Indirect sandwich ELISA and indirect antigen coating ELISA, respectively.

et al., 1997; Lea et al., 1994). Afterwards, Freiberg et al., (2001), utilized VP2 specifically raised MAb and elucidated that some internal domains of the capsid proteins were displayed to the surface through the structural flexibility properties, enabling them to be antigenic sites. The rVP2 protein was initially injected intraperitoneally followed by four consecutive intravenous boosters. One-week following the fourth intravenous injection, sera were collected, mice were euthanized and spleens were collected for RNA extraction followed by cDNA synthesis by reverse transcription. Subsequently, repertoire of V_L and V_H coding regions were PCR-amplified from cDNA (Fig. 4A). The resulting amplicons were assembled through joining the 3′- end of V_H amplicons with the 5′- end of V_L using the SOE-PCR method (Fig. 4B). A phage library was constructed by cloning of the assembled scFv repertoire into the *SfiI* site of $P^{ADL-22c}$ phagemid vector. Proper production of the recombinant $P^{ADL-22c}$ phagemid was verified by *SfiI* digestion of its DNA exhibiting scFv insert of the expected size (Fig. 4C). After three rounds of panning against rVP2, the size of scFv phage library obtained in this study was $\sim 1.9 \times 10^8$ pfu which was in accordance to scFv libraries obtained in other studies (Crowther et al., 1993; Logan et al., 1993). It should be noted that the successful isolation of a desirable scFv from a phage display library depends on size of the library. In turn, that is based on quality of isolated RNA, cDNA construction and efficiency of transformation and selection.

Eight distinct scFv clones were selected from the last round of panning and subjected to further characterization and validation. It is noteworthy mentioning that in the V_H and V_L regions of antibodies there are three areas of increased variability, called hypervariable regions or complementarity-determining regions (CDR1, CDR2, and CDR3). Between those CDRs are four regions of more conserved sequences called framework residues (FWs). The CDRs in V_H and V_L are short segments (7–17 amino acid residues each) and collectively they constitute the antigen-binding sites of antibodies, whereas the FWs support the binding of the CDR to the antigen and stabilize the CDR structure. It was also suggested that FWs act as a polypeptide scaffold, which enable the CDRs to fold correctly and to expose on surface of the variable chains so that it will be accessible for antigen recognition and binding. Among CDRs domains, CDR3 is the most variable in terms of amino acid composition and length (Chothia et al., 1989). Interestingly, the eight selected clones in our study were highly variable in the CDR3 and CDR1of V_H and V_L, respectively (Figs. 5 and 6).

Selected scFv clones were transformed to a non-suppressive amber codon (UAG) *E. coli* SS320 for expression. The UAG stop codon was introduced in the recombinant phagemid to turn off expression of the P^{III} fusion protein by switching to a non-suppressor strain of *E. coli*, such as SS320, allowing only the production of soluble scFv without further sub-cloning. After induction, periplasmic proteins were purified and separated on SDS–PAGE (Fig. 7A). All clones produced distinct polypeptide of an apparent molecular weight of ~27 KDa (Fig. 7A, arrow), which was absent in the periplasmic extract of negative control (Fig. 7A, lane 2). The 27 KDa represented a fusion polypeptide that comprised the His tag and a scFv polypeptide. The identity of putative 27 KDa was confirmed by Western blot assay using anti-His tag antibody (Fig. 7B). Furthermore, Ni-NTA affinity resins were used to purify the recombinant His-scFv proteins from periplasmic extract. Moreover, we were able to purify scFv near homogeneity (Fig. 7C). Following purification, it was necessary to assess the proper folding of scFv as antibody and to ensure that the proteins did not lose its recognition and binding properties to the rVP2 during purification. Indirect ELISA (Fig. 8) showed that all clones gave very strong signals, especially Mab3c50.

The affinity detection of developed scFv for FMDV serotypes was confirmed through two different methods of ELISA, the indirect ELISA and indirect double antibody sandwich ELISA (Fig. 9). Our data showed that two clones, Mab1c1 and Mab3c50, were highly potent in

recognising the three FMDV (O, A, and SAT 2) serotypes under investigation. Two clones, namely Mab3c14 and Mab3c16, reacted more strongly to SAT 2 than serotypes A and O. Mab1c62 was feebly reactive.

Diagnostic methods based on isolation of FMDV from clinical specimens of infected animals are time-consuming and laborious and must be followed by an immunoassay for its identification (Knowles and Samuel, 2003; Elgaied et al., 2017; Salem et al., 2018). This study illustrated the production of a novel group of recombinant scFvs for serotype-independent diagnosis of FMDV, where previous efforts for the development of a double antibody sandwich ELISA based on the seven serotype-specific polyclonal antisera derived from FMDV-immunized rabbits and guinea-pigs, have been developed (Have et al., 1984; Roeder and Le Blanc Smith, 1987). Further, a serotype-specific MAb-based ELISA (Samuel et al., 1991) and a competitive ELISA using MAb specific for the 12S subunit protein from six of the seven serotypes were developed for the detection of FMDV (Smitsaart et al., 1990). Also, DAS-ELISA based on two MAbs with different specificities (Yang et al., 2007) and an assay based on MAbs produced through phage-displayed screening (Liu et al., 2017), were recently proposed for type-independent FMDV diagnosis. Based on results of this endeavor, the developed recombinant scfv MAbs might be a promising diagnostic reagent as an alternative to the costly serotype-specific guinea-pig or rabbit antisera; as it self and enzyme label-conjugated can be utilized as capture antibody and detecting antibody, respectively.

Specificity of the developed VP2 recombinant scfvs MAb was insufficiently recognized as one strain per three FMDV serotypes, were merely studied. Frankly, we studied the available local isolates/strains in Egypt as strict legislations prevent introduction of any foreign strains across borders. That might be resolved by a follow up work in collaboration with an international reference FMD laboratory. Besides, future work is required to identify the VP2 epitopes that are specific to the eight produced recombinant scfvs as well as to evaluate their reactivity and specificity to the 146S (intact FMD virion particle) antigen. That would be of significant use in 146S based quality control testing of FMDV inactivated vaccines. However, there is experimental evidence that the similar level of neutralizing antibody against the first FMDV was stimulated by the heterotypic 146S particles and homotypic 146S particles. This displayed a similarity in the antigenic structure among the different serotypes and might sharply reduce the sensitivity of ELISA, which antibodies to 146S were adopted in (Cartwright et al., 1980).

In summary, FMDV is enzootic in Egypt with the prevalence of the three serotypes O, A, and SAT 2. Thus, we focused on developing molecular tools that detect these particular serotypes in sera of infected animals along with possible use of such tools for emergency immunization. To that end, a panel of FMDV SAT 2 derived scFv was produced and a phage display library (1.9×10^8 pfu) was constructed and panned against rVP2 capsid protein. Highly specific scFv candidates were obtained and validated. We believe that the scFv obtained during this study have the potential to provide a prompt diagnostic tool at the ports, which considered to be a crucial step in preventing the entrance of imported infected/carrier animals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.012.

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