ORIGINAL PAPER

Removal of 3C protease from the 3ABC improves expression, solubility, and purification of the recombinant 3AB of foot-and-mouth disease virus

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Abstract

During an ongoing outbreak of Foot-and-Mouth Disease Virus (FMDV), it is crucial to distinguish naturally infected from vaccinated seropositive animals. This would support clinical assessment and punctual vigilance. Assays based on 3ABC non-structural protein as an antigen are reliable for this intention. However, the insolubility and degradation of recombinant 3ABC during expression and purification are serious challenges. In this study, alternatively to expressing the recombinant 3ABC (r3ABC), we expressed the 3AB coding sequence (~672 bp) as a recombinant protein (r3AB) with a molecular mass of ~26 KDa. Analytical data from three-dimensional structure, hydrophilicity, and antigenic properties for 3ABC and 3AB exhibited the 3C protein as a hydrophobic, while 3AB as a hydrophilic and highly antigenic protein. The expressed r3AB was recovered as a completely soluble matter after merely native purification, unlike the full expressed r3ABC. Immunoreactivity of r3AB to anti-FMDV antibody in infected sera with different FMDV serotypes was confirmed by the western blot and indirect ELISA. Besides, the authentic antigenicity of purified r3AB was demonstrated through its ability to induce specific seroconversion in mice. Summarily, the removal of 3C: has influenced neither 3D structure nor antigenic properties of the purified r3AB, overcame insolubility and degradation of the r3ABC, and generated a potential superior antigen (r3AB) for herd screening of animals to any FMDV serotype.

Keywords FMDV · Non-structural 3ABC · 3C removal · Bacterial gene expression · Insolubility · Immunoreactivity · Recombinant 3AB

Introduction

Foot-and-mouth disease virus (FMDV) is causing the most critical viral infection attacking livestock and restricting the international trade of live animals and animal products. Outbreaks of FMDV have been unending even though vaccination strategies are applied. Persistence of asymptomatic infection can be established among most ruminants (with the exception of African buffalo) following the recovery causing continuous virus replication and shedding [1]. Furthermore, immunization of livestock with inactivated vaccines

is applied in enzootic regions to control FMDV circulation as a part of the eradication programs [2]. This immunization approach stimulates the production of antibodies indistinguishable from those produced due to infection after exposure to the live FMDV. However, use of purified FMDV in preparation of inactivated vaccines will get rid of NSP of FMDV, allowing differentiation between anti-FMDV antibody responses from vaccinated and animals infected with FMDV (antibody response to both SP and NSP and proteins). Distinguishing FMDV naturally infected from vaccinated in animals, is still necessary for an early outbreak warning, medical inspection, during export and import of livestock and their products [3].

FMDV genome encodes a single polyprotein that is split by viral proteases and cotranslational processing by cellular proteases into distinct polypeptides including several different non-structural proteins and four structural proteins [4, 5]. FMDV infection elicits antibodies (Abs) against all viral antigenic components represented by structural proteins (SPs) and non-structural proteins (NSPs), whereas

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vaccination with purified FMDV vaccines elicits only anti-SPs Abs with a lack of anti-NSPs [6]. Accordingly, several FMDV's NSPs have been utilized as candidate antigens to serologically differentiate infected from vaccinated animals, with the superiority of the non-structural protein (NSP) 3ABC [6–10]. It has been reported that anti-3ABC Ab could be early detected after FMDV infection and remain circulating in cattle longer than other anti-NSPs Ab [11–13].

Escherichia coli harboring 3ABC coding sequence usually produce low yield and insoluble recombinant protein, which seems to be a sequel of 3C presence [14]. The FMDV 3C protein is a major viral protease that cleaves 10 of 13 cleavage sites within the viral polyprotein [15, 16], and could even fully cleave the expressed protein [17]. In addition, the high content of hydrophobic regions in the polypeptide may lead to protein instability, aggregation and finally forming the inclusion bodies [18, 19].

It is possible to obtain a soluble recombinant protein by strategies that amend the factors leading to inclusion bodies formation [19, 20]. Failing to express the native full-length 3ABC polyprotein in different expression systems (Baculovirus and *E. coli*) was reported [21]. That have been overcame by the insertion of point mutations to substitute two out of three active protease sites in 3C, then the mutated 3ABC polyprotein was used effectively for the differentiation of FMDV infected and vaccinated animals (DIVA) [21].

This work aimed to explore the significance of 3C removal from the NSP 3ABC of FMDV on properties of the remaining 3AB protein, regarding its solubility and antigenicity. That would optimize the production of an intact soluble and reliable antigen, as a versatile alternative to 3ABC, for economic serosurveillance and accurate recognition of infected amid vaccinated and naive animals (DIVA) during FMDV outbreaks as well as at import-export quarantine.

Materials and methods

Viral RNA

Total RNA was extracted from baby hamster kidney (BHK$_{21}$-clone 13) cells infected with FMDV serotype SAT 2 isolate (Egy/2012) using the SV-total RNA isolation system

(Cat.# Z3100). FMDV has been propagated at the Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, where BHK$_{21}$ cell line has been maintained in a minimum essential medium with Eagle's salts (MEME) supplemented with 100 U/mL penicillin, heat-inactivated 5–10% newborn calf serum (NCS), 25 i.u./mL mycostatin and 100 μg/mL streptomycin.

Gene isolation and cloning

For cDNA synthesis, the above isolated RNA was used as a template. The 3ABC and 3AB coding sequences were subsequently amplified with polymerase chain reaction (PCR) from synthesized cDNA. Primers for 3ABC and 3AB isolation were designed based on the FMDV serotype SAT 2 available sequences retrieved from the NCBI database and using Oligo 6.0 and DNASTAR software. One forward primer (served for both 3AB and 3ABC) and two reverse primers (one for each) were used. The locations and sequences of the specifically designed primers are shown in Fig. 1 and Table 1, respectively. PCRs have been performed under the following conditions: for 3ABC, 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, while the same conditions were used for 3AB except for the extension time that was diminished to 30 s.

The amplified 3ABC and 3AB PCR products were purified by QIAquick Gel Extraction Kit (Cat.# 28,704), Qiagen. They have been consequently cloned in the P$^{GEM-T-easy}$ vector, Promega, and propagated into *E. coli* (Top10) as was instructed by the manufacturer. Recombinant plasmids were mini-prepared using GeneJet Plasmid Miniprep Kit (Cat.# K0502); and after sequence confirmation, cloned fragments were sub-cloned into different expression vectors (P$^{QE80L-Kan}$,

Table 1 Specific sequences of designed PCR primers used for isolation of FMDV NSP 3ABC and 3AB coding sequence

Primer sequence[a]	ID	Restriction enzyme
ggatccatctcgattccttcccaaaagtccg	1	*Bam*HI
gtcgacctcagtgacaatcaagttcctagc	2	*Sal*I
gtcgacctcgtggtgcggttcagggtc	3	*Sal*I

[a]*Italic* and bold sequences show the added restriction sites for downstream sub-cloning procedure
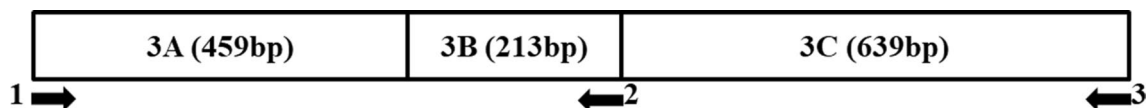

Fig. 1 An indicative diagram for locations of the designed primers spanning the FMDV NSP 3ABC polyprotein coding sequence; Arrows 1 and 2 are for forward and reverse specific primers, respectively, for isolation of the 3AB fragment (~672 bp); Arrow 3 shows location of the reverse primer that works with forward primer of Arrow 1 for full 3ABC isolation (~1311 bp)

P$^{Gex-4t1}$, and P$^{ET30a(+)}$). After transformation in different host *E. coli* strains (Bl21DE3Rill, Bl21DE3, and TG1), the resultant positive recombinant clones were selected on suitable antibiotic agar plates. Grown clones were screened using the same primers and conditions of the original amplifying PCR for the presence of the inserts.

Gene expression

Experiments for 3AB and 3ABC gene expression were designed in accordance with our established procedures [22–24]. A positive clone from each sub-cloning (P$^{QE80L-Kan}$, P$^{Gex-4t1}$, and P$^{ET30a(+)}$), individually transformed into three different host *E. coli* strains Bl21DE3Rill, Bl21DE3, and TG1, was tested for expression. In brief, an overnight culture of a bacterial clone containing recombinant plasmid was inoculated into a fresh LB broth medium supplemented with the desired antibiotic/s at 37 °C till OD$_{600}$ reached 0.6. Various concentrations of IPTG (0.1, 0.5, and 1 mM) for induction, and re-incubation at various temperatures (4, 16, and 37 °C) for additional different periods (2, 3, 4, and 16 h), were applied for each construct in each *E. coli* strain. Consequently, the bacterial cells in grown cultures were harvested by centrifugation at 4000×g for 20 min and re-suspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0). For complete lysis, the cells were exposed to one freeze-thaw cycle (−80 °C/37 °C), followed by sonication on ice for a few seconds.

Analysis of protein expression

Bacterial lysates containing expressed proteins were analyzed by SDS-PAGE. Furthermore, western blotting was used to assess the quality and quantity of protein expression; as well as estimating the specific immunoreactivity of expressed recombinant proteins. Briefly, on SDS-PAGE, total lysate proteins were separated, and then blotted to a nitrocellulose membrane. Residual membrane-spaces were blocked by 4% BSA overnight at 4 °C. Mice antibody against 6xHis was diluted in TBS and added to the membrane after its washing with TBS-Tween 0.1% for 2 h with gentle shaking at room temperature. As before, the membrane was washed, and the ALP-conjugated anti-mouse (Sigma, USA), diluted 1:20000, was added for 1 h at room temperature as a secondary antibody. The membrane was washed as such before. Color-reaction was developed by a formulated solution containing NBT and BCIP substrates.

Protein modeling structure, hydrophilicity and antigenicity

Three-dimension structure was modeled with I- Tasser software for 3ABC and 3AB protein-coding sequences, and Pymol software was used to view modeling results. The Protean program of DNALaser software was used to reveal the hydrophilic and hydrophobic regions, respectively; the antigenicity properties before and after removal of the 3C protein-coding sequence, were also identified using the same software.

Solubility determination of recombinant proteins

To assess the solubility or insolubility of recombinant expressed protein, LB medium (10 ml) containing 100 µg/ml suitable antibiotic was inoculated with a bacterial clone containing the recombinant plasmid harboring the target coding sequence. After shaking at 37 °C overnight, pre-warmed media (with required antibiotic/s) were inoculated with 2.5 ml of the overnight growth and re-grown at 37 °C with intense shaking (~300 rpm) until the OD$_{600}$ was 0.6. Protein expression was induced by adding 1 mM IPTG, and then the culture was re-incubated at 16 °C for 4 h. Cells were collected by centrifugation; the pellet was re-suspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mMNaCl, pH 8.0), followed by adding Lysozyme (1 mg/ml) for 30 min on ice. The lysate was exposed to 6 × 10 s with 10 s pauses of sonication on ice at 200–300 W. Lysate was then centrifuged for 20–30 min at 10,000×g at 4 °C. The supernatant was decanted, and the pellet was re-extracted a few times with 0.25% tween 20 and 0.1 mM EDTA. The above mentioned SDS-PAGE and western blotting analysis were applied to analyze all extracted fractions.

Purification of recombinant 3AB

For purification of recombinant 3AB (r3AB) protein, a 200 ml overnight culture from a selected clone harboring the recombinant plasmid P$^{QE80L-Kan-3AB}$ was centrifuged, and the total proteins were extracted as described above. 3AB peptide fused with N-terminal 6xHis-tag was purified from the re-suspended pellet using Ni-NTA agarose resin (Qiagen, Germany) as was described in the manufacturer's instructions. Finally, eluted recombinant 3AB-6xHis containing imidazole was subjected to dialysis against 1x PBS overnight at 4 °C, and its concentration was measured using Bradford protocol as previously described [25, 26].

Assessment of 3AB antigenicity

The antigenicity of expressed r3AB protein was assessed through raising specific antibodies in mice. Six-week-old mice from Theodor Bilharz Research Institute were accommodated in a pathogen-free environment for 5 weeks duration and given access to food and water. The procedures and policies of the National Institute of Health (NIH) have been followed for handling mice; in addition, the experiment has been approved by the Animal Care and

Use Committee in Veterinary Medicine, Cairo University (Vet CU16072020180). Mice have injected intraperitoneally with 30 µg recombinant r3AB protein emulsified with Freund's complete adjuvant for one time, followed by four injections (50 µg/each) of recombinant 3AB protein emulsified with incomplete Freund Adjuvant, weekly administered. Daily, mice were observed, and serum was collected every week before injection. By the end of the immunization protocol, mice were euthanized humanely for serum collection.

A 96-well ELISA plate, Nunc, Denmark was coated with the recombinant 3AB protein (50 ng/well) in carbonate and bicarbonate buffer (pH 9.6) at 4 °C/overnight. The wells were washed with PBST. Residual-spaces were blocked with BSA (3%), followed by washing again with PBST. Collected serum (before each booster) was diluted 1:3000 in PBS and added for 2 h at room temperature. The zero-time mice serum (prior to the first mice-immunization) was collected and used as a negative control. The wells were intensively washed with PBST. Rabbit Anti-mouse IgG conjugated with ALP (Sigma Aldrich) was applied by a dilution 1:20000 at room temperature for 2 h. The excess and unbound conjugated antibodies were removed by washing with the phosphate buffer saline-Tween (PBST), and 100 µL of p-Nitrophenyl Phosphate (PNPP)-substrate solution was used to develop the reaction. NaOH (3 M) has been added to stop the reaction before measuring the absorbance at 450 nm using the I-Mark microplate reader, Bio-Rad.

Assessment of recombinant 3AB immunoreactivity

The recombinant 3AB protein (r3AB) immunoreactivity with the FMDV antibodies (Ab) in FMDV infected serum was assessed. The specificity of r3AB protein to detect the anti-FMDV Ab in the serum of infected animals was analyzed by an indirect ELISA, which was practically performed as above mentioned. Purified r3AB protein (50 ng/well) was coated in ELISA plates. As a recombinant VP2 protein developed in our lab [27] has been effectively used earlier for anti-FMDV Ab detection, it was used herein by the same concentration in parallel with r3AB as a coating antigen. Sera from naïve calves infected with FMDV O, A, and SAT 2 serotypes, have been separately mixed in PBS containing 3% BSA, plated at 100 µL per well in duplicate (four times) and incubated for 2 h at 37 °C. After pouring the sera and washing as previous, anti-bovine IgG (KPL, MD, USA) labeled with horseradish peroxidase diluted at 1:1000 in PBS containing 3% BSA was added (100 µL/well). After incubation at 37 °C for 1 h, the ELISA plate was washed, and the TMB solution (100 µL per color well) was added to develop a colorimetric reaction.

Results

Isolation, cloning and expression of 3ABC coding sequence

RT-PCR using the total RNA from BHK_{21} cells infected with FMDV serotype SAT 2 isolate Egy/2012 and the 3ABC specific primers (1 & 3), produced a discrete band of ~1300 bp correspondent to the full 3ABC coding sequence. On the other hand, RNA from non-infected BHK21 cells did not amplify any detected band (Fig. 2a).

Protein expression of the isolated 3ABC was optimized through using different expression vectors, host *E. coli* strains, temperatures, and time of incubation as well as IPTG concentrations. As these factors were modified, a slight change in the level of recombinant protein expression was observed (data not shown). Summarily, the optimal expression was obtained when the 3ABC coding sequence was sub-cloned into a $P^{Gex-4T1}$ expression vector in frame with GST-tag, followed by transformation in BL21(DE3) and induction of protein expression with 1 mM IPTG at 16 °C for 4 h. Subsequently, after transformation of the recombinant vector harbored the 3ABC ($P^{Gex-4T1-3ABC}$), the resulting recombinant BL21(DE3) clones were screened using the original specific PCR (data not shown). A clear expression of 3ABC-GST-fusion polypeptide (~78 KDa) in the cell lysate from positive bacterial clone was noticed (Fig. 2b) compared to the negative control (lysate of the same cells transformed with non-recombinant $P^{Gex-4T1}$ expression vector).

Expression of the recombinant 3ABC (r3ABC) fused with GST was confirmed by western blot using anti-GST Ab. The results showed unique detection of r3ABC fused to GST as an apparent band (~78 KDa) in the cell lysate of BL21(DE3) transformed with recombinant $P^{Gex-4T1-3ABC}$ expression vector that was absent in cell lysate of the same bacteria transformed with the non-recombinant vector (Fig. 2c).

Solubility determination of the expressed r3ABC polyprotein

After several trials for purification using different protocols under native conditions, the r3ABC was repeatedly precipitated in an insoluble matter (data not shown). To assess whether it is insoluble or only associated with membrane fragments of the cell pellet, cells containing the r3ABC were fully lysed by 0.25% Tween 20, 0.1 mM EDTA for multiple times. The test has confirmed that the r3ABC is a fully insoluble protein (Fig. 3a), as nearly all the quantity of r3ABC has been precipitated in the
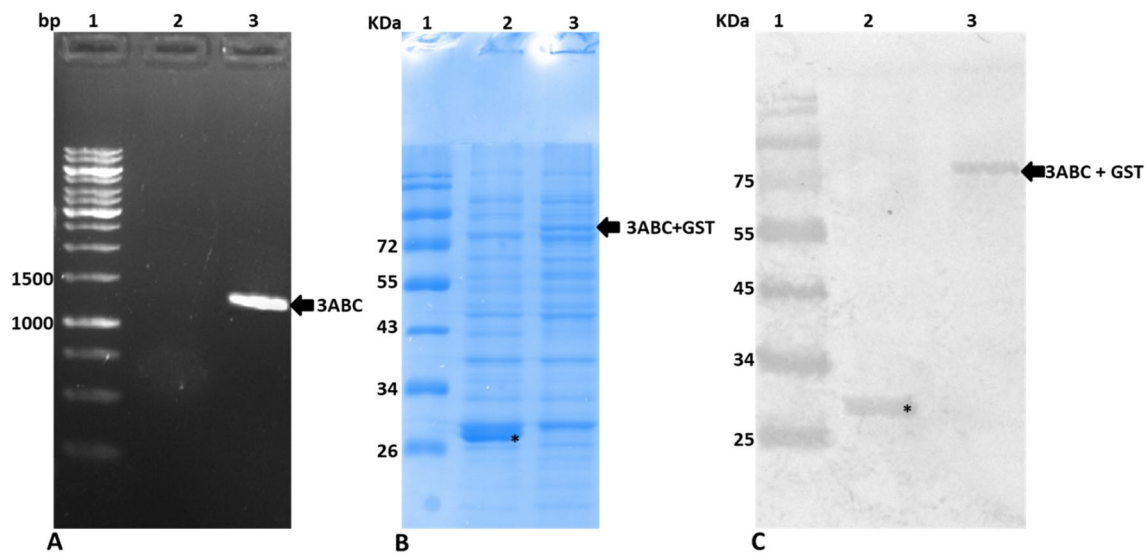
Fig. 2 RT-PCR amplification and protein expression analysis for r3ABC. (**a**): 1.2% Agarose gel electrophoresis of RT-PCR amplification for 3ABC coding sequence. 1: 1 kb DNA ladder, 2: Negative control (RNA from non-infected BHK_{21} cells), 3: The 3ABC amplicon at its expected size of ~1300 bp (RNA from FMDV SAT2 infected BHK_{21} cells). (**b**): SDS-PAGE analysis of cell lysate from BL21DE3 transformed with expression vector, separated on 12% denaturing polyacrylamide gel and stained with 0.25% Coomassie Brilliant Blue R 250 solution, Lanes: (1) Pre-stained molecular weight protein marker; (2) lysate from BL21DE3 transformed with non-recombinant $P^{Gex-4T1}$ showing expression of GST at its expected size of ~26 kDa (steric); (3) lysate from BL21DE3 transformed with recombinant $P^{Gex-4T1-3ABC}$ revealing expression of r3ABC fused to GST at its expected size of ~78 KDa. (**c**): Western blot analysis for r3ABC expression in BL21DE3, Lanes: (1) Pre-stained molecular weight protein marker; (2) Recombinant GST peptide recovered from lysate of BL21DE3 transformed with non-recombinant $P^{Gex-4T1}$ and reacted to anti-GST Ab (~26 KDa, steric); (3) r3ABC fused with GST lysate recovered from lysate of BL21DE3 transformed with recombinant $P^{Gex-4T1-3ABC}$ and reacted to anti-GST Ab (~78KDa). (Arrows denote to sizes of the expressed recombinant proteins)
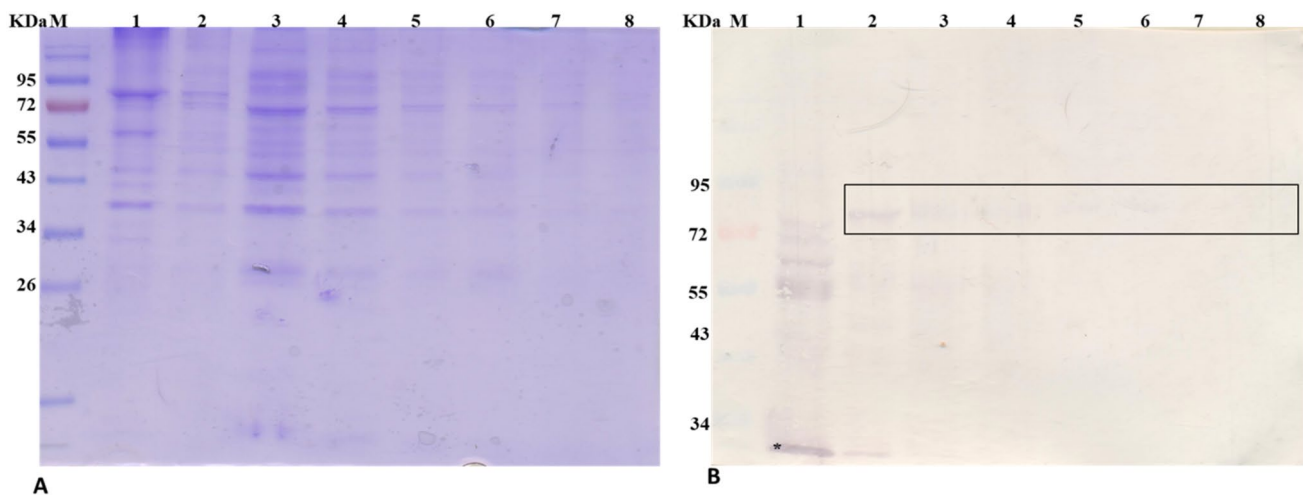

Fig. 3 Solubility test for expressed 3ABC. (**a**): SDS-PAGE analysis representing trials to purify the r3ABC from BL21DE3 cell lysate transformed with recombinant $P^{Gex-4t-1-3ABC}$ vector. The r3ABC was almost precipitated in pellet (1), small amount in flow-through (2), and completely invisible in the rest of purification fractions (3–8). (**b**): Western blotting for detection of the r3ABC fused with GST using anti-GST antibody. No signals were detected in cell lysate from bacteria transformed with non-recombinant $P^{Gex-4t-1}$ vector unless a low molecular weight band (steric) revealing only expression of GST (1); high signals were detected in pellet fraction (2), while low signals (boxed) were detectable in some of other purification fractions (3–8)

pellet. A little amount was visible in flow-through, and an extremely low amount was gained from different fractions which could not be visualized using SDS-PAGE analysis. However, it could be detected using anti-GST Ab in the western blot analysis (Fig. 3b), as the r3ABC precipitated in the pellet fraction was distinguished with high signals, while the other fractions displayed extremely low signals. No detected signals in cell lysate from the transformed bacteria with non-recombinant P$^{Gex-4T1}$ vector, except a low molecular weight band (~26KDa) indicating the expression of the non-fused GST peptide.

## Effect of 3C removal on 3D structure, hydrophilicity and antigenicity properties

To understand the reasons of r3ABC's insolubility, we have studied some of 3ABC properties before and after the removal of 3C coding sequence. Three-dimensional (3D) structure for 3ABC and 3AB coding sequences, was predicted in spherical shape (Fig. 4). Interestingly, we found that the removal of 3C protein-coding sequence does not affect the 3D structure of 3AB protein. Moreover, the 3ABC hydrophilicity analysis revealed that 3C coding region is a hydrophobic protein, while 3AB region is hydrophilic one. Moreover, studying the antigenicity properties showed that the removal of 3C would not affect the antigenic properties of 3AB which appeared as a highly antigenic protein compared to the 3C (Fig. 5).

3AB isolation, cloning and expression

Upon its above promising properties, we have isolated, cloned and expressed 3AB after we omitted the 3C coding region. RT-PCR using total RNA from BHK$_{21}$ cells infected with FMDV serotype SAT 2 isolate Egy/2012 and specifically designed primers for 3AB (1 & 2) amplified a corresponding band to the 3AB (~672 bp) coding sequence (Fig. 6a). DNA of recombinant P$^{Gex-4T1-3ABC}$ and RNA of non-infected BHK$_{21}$ cells, were used as positive and negative control, respectively.

Repetitively, the abovementioned conditions used for 3ABC expression were applied to optimize the 3AB protein expression. A small difference in the intensity of recombinant 3AB expression band was detected as conditions were varied. Summarily, the best expression results were obtained when 3AB coding sequence was subcloned into P$^{QE-80L-Kan}$ expression vector in frame with the His-tag, followed by transformation in BL21(DE3) and expression was induced with 1 mM IPTG at 16 °C for 4 h. Subsequently, the resultant recombinant clones were picked up from Kanamycin LB agar plates and screened by PCR (not shown). A high level expression for 3AB-His-fusion polypeptide was produced from a bacterial positive clone (Fig. 6b). Subsequently, western blotting analysis (Fig. 6c) for cell lysate reacted to anti-His-Ab confirmed high expression of 3AB-His-fused recombinant protein.

Fig. 4 Computational modeling of the 3D structure in sphere shape for the 3ABC and 3AB protein in A and B, respectively. Colors: blue, firebrick, and tv-green are showing 3A, 3B, and 3C, respectively
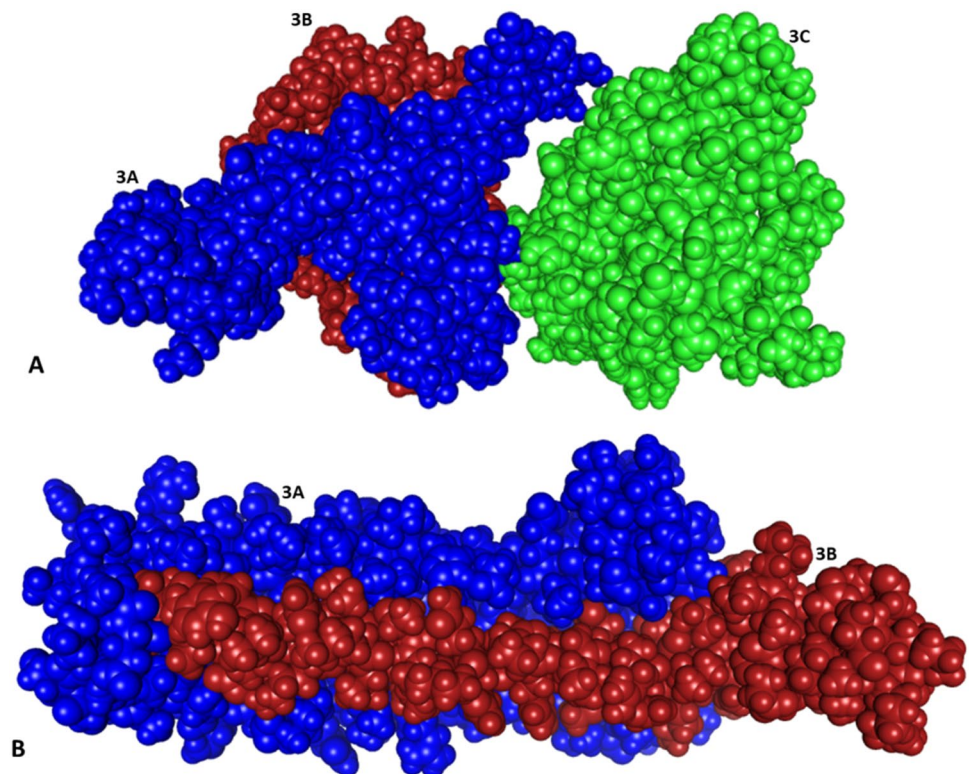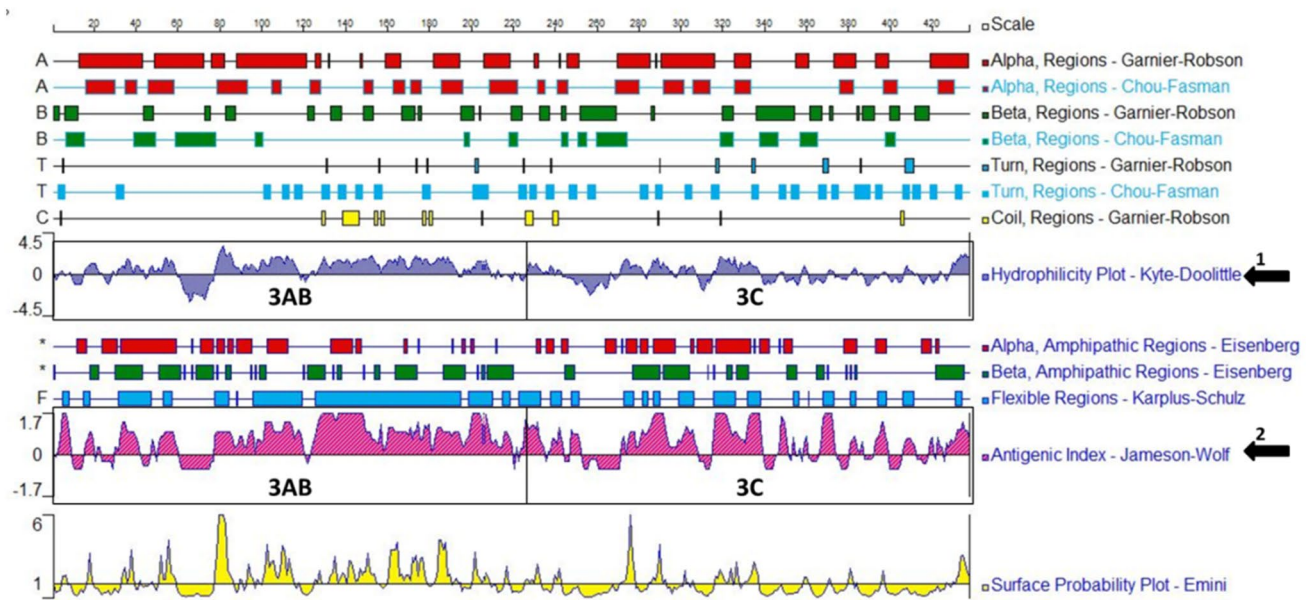
Fig. 5 Analyses by protean, DNA Lasergene software for some of 3ABC properties. Hydrophilicity analysis (arrow 1) revealed 3AB as a hydrophilic protein, while 3C appeared as a highly hydrophobic protein. Moreover, 3ABC antigenicity (arrow 2) demonstrated 3AB as a strong antigenic protein
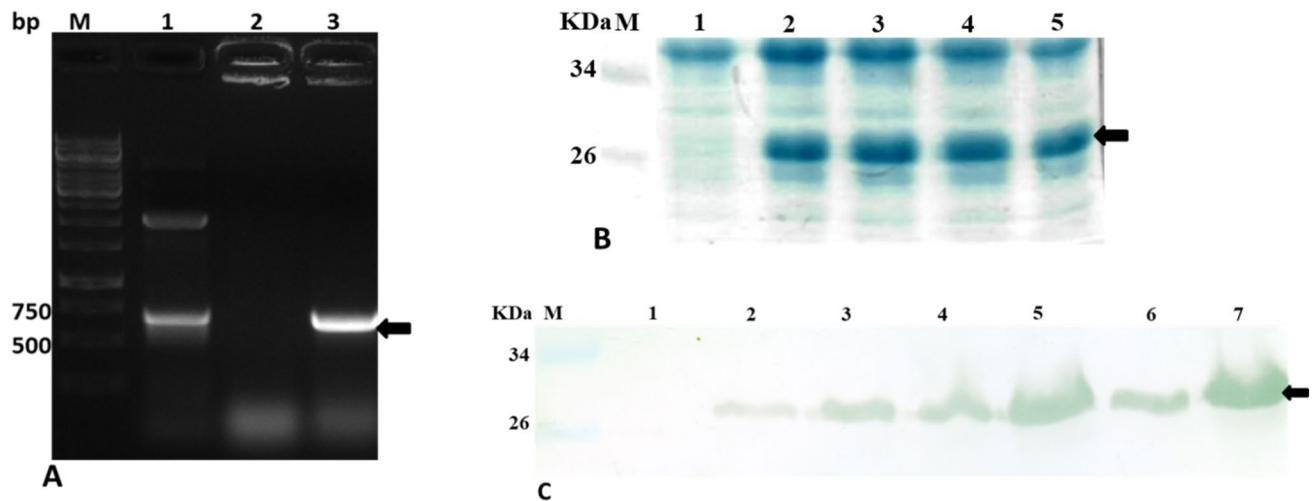

Fig. 6 RT-PCR amplification and protein expression analysis for r3AB. (**a**): Agarose gel electrophoresis for the RT-PCR amplicons of FMDV 3AB coding sequences, stained with ethidium bromide. Lanes: (M) 100 bp DNA size marker, (1) DNA from recombinant $P^{Gex-4T1-3ABC}$ vector was used as a positive control, (2) Total RNA from non-infected BHK_{21} cells used as a negative control, (3) RNA from BHK_{21} cells infected with FMDV SAT2 isolate Egy/2012 showing discrete 3AB amplicon at the expected size of ~672 bp. (**b**): 12% denaturing SDS-PAGE of r3AB expression. (M) Pre-stained molecular weight protein marker, (1) Clear lysate from BL21DE3 transformed with non-recombinant $P^{QE-80L-Kan}$ expression vector (as a negative control), (2–5) Clear lysates from different BL21DE3 clones transformed with recombinant $P^{QE-80L-Kan-3AB}$ revealing expression of r3AB protein fused to His-tag at its expected size of ~26 kDa. (**c**): Western blot analysis showing the detection of fused r3AB-His recombinant peptide using anti-His Ab. (M) Pre-stained molecular weight protein marker, (1) Clear lysate from of BL21DE3 transformed with non-recombinant $P^{QE-80L-Kan}$ expression vector showing no signals (as a negative control), (2–7) The r3AB fused with His-tag at its expected size of ~26 KDa in clear lysates of different BL21DE3 clones transformed with recombinant $P^{QE-80L-Kan-3AB}$. (Arrows denote to sizes of the expressed recombinant proteins)

Purification of recombinant 3AB protein under native conditions

The r3AB fused to His-tag was purified using Ni-NTA affinity chromatography, eluted, and the fractions have been analyzed (Fig. 7). A protein size of ~26 KDa was clearly visible as a single pure band. Remarkably, we found that r3AB was not unlike most recombinant proteins as it was expressed
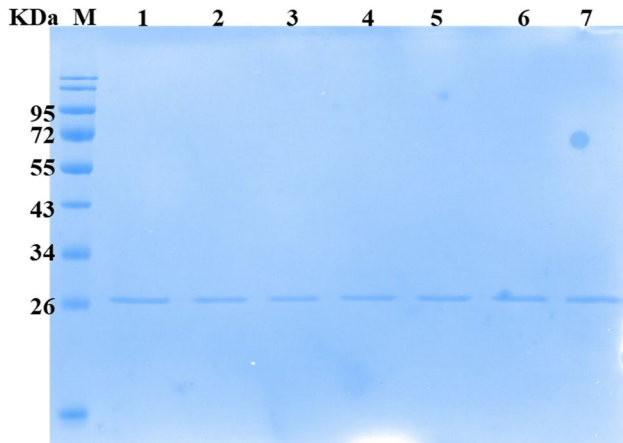

Fig. 7 SDS-PAGE analysis of the clear purified r3AB tagged with His protein. Lanes (1–7): Different fractions eluted throughout the affinity chromatography purification using Ni-NTA beads

within a fully soluble material, thus native purification procedure was adequate for its recovery, and protein folding procedure was unnecessary. Noticeably, the eluted fractions did not contain other proteins rather than the 6xHis-r3AB due to extensive imidazole washes. Accordingly, it was unnecessary to perform additional SDS-PAGE purification or electro-elution before using the purified 3AB for any further application. Different batches of r3AB were prepared and purified that began with about 1.5 g of *E. coli* cells obtained from 200 ml culture. The purified r3AB yield has been measured on frequent basis as an approximate of 4 mg per each liter of medium.

Immunoreactivity and immunogenicity of recombinant purified 3AB

Immunoreactivity of purified r3AB against FMDV antibodies in serum from FMDV infected animals was assessed by an indirect ELISA. Significant positive signals were observed when purified r3AB fused to 6xHis was used as a coating antigen. As recombinant VP2 protein (developed in our lab) was effectively used earlier for FMDV antibodies detection, it was used in parallel with r3AB as a coating antigen. Moreover, as the 3AB coding sequence is highly conserved between the different FMDV serotypes, the three different FMDV serotypes (A, O, and SAT 2) antibodies
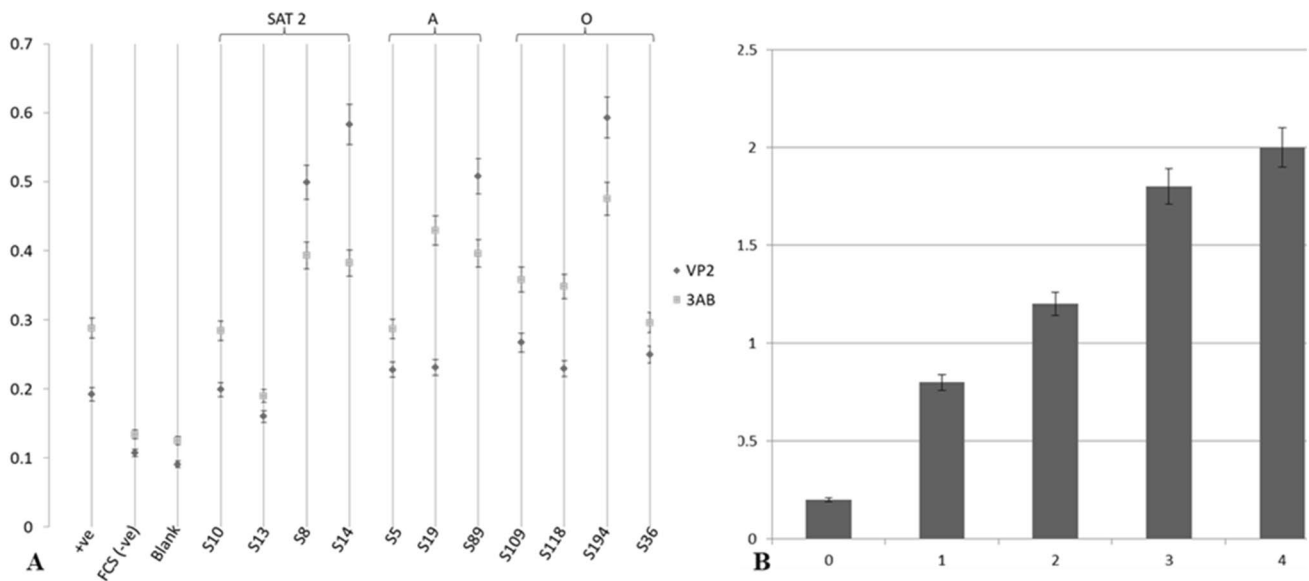

Fig. 8 Evaluating the immunoreactivity and immunogenicity of purified recombinant 3AB. (**a**): Indirect ELISA showing the detection of anti-FMDV Ab in sera from infected animals. Eleven samples were tested; four of them are infected with serotype SAT2, 3 with serotype A, and finally 4 with serotype O. All infected sera were reacted positively with r3AB and also with VP2 recombinant protein which was used in parallel with r3AB as a coating antigen. A positive FMDV certified sera, and FCS were used as positive and negative control, respectively. (**b**): Indirect ELISA showing the elevated immune response against r3AB in immunized mice serum. A significant increase in binding activity to the given antigen (r3AB) with serum samples collected at 0, 1, 2, 3, and 4 weeks post initial immunization. The error bar represents 5% more or less of the ELISA reading value

in infected sera were reacted positively with 3AB antigen (Fig. 8a).

Besides, the purified r3AB immunogenicity was demonstrated by its ability to raise anti-FMDV 3AB Ab in mice serum, where ELISA tested the precise seroconversion of mice against the purified r3AB. ELISA detected a reasonable seroconversion in immunized mice sera with a significant sequential increase in the reactivity to the coating antigen (r 3AB) with the sera collected on 1, 2, 3, and 4 weeks post initial immunization (Fig. 8b).

Discussion

In enzootic regions like the Middle East including Egypt, the national vaccination program using inactivated vaccines is an essential control measure against FMDV outbreaks that are endlessly emerged. The occurrence of asymptomatic and subclinical FMDV infections within a herd followed by recovery can establish persistent virus replication and shedding. Therefore, serological herd testing should be applied to recognize such persistently infected and shedder animals. Thus, earlier studies [7, 28] have been proposed using nonstructural proteins (NSPs) as FMDV infection markers, putting into consideration that recent inactivated vaccines stimulate antibody (Ab) response against merely the structural proteins (SP) of FMDV. Consequently, the potential use of the NSPs for differential detection of FMDV infected animals amid vaccinated herds was well established and several NSPs have been exploited as antigens for this purpose [6, 7, 11, 29–33]. The most appropriate antigen for such serological monitoring has been the recombinant 3ABC polyprotein. This significant superiority of the 3ABC-based assays has been illustrated earlier [11–13], as the anti-3ABC Ab could be identified earlier post-infection and remained detectable in circulation longer than other anti-NSP Abs.

Expression of the 3ABC has been reported in *E. coli*-based systems [6, 11–13, 28, 34]. However, those studies did not introduce any crucial details for the successful 3ABC full-length expression as most of these attempts met the insolubility hurdles of 3ABC production. In the current work, we faced the same problem, as the 3ABC encoding sequence (~1300 bp) was isolated by RT-PCR utilizing genomic RNA of FMDV serotype SAT 2 isolate Egy/2012 extracted from virus-infected BHK_{21} cells. The expressed recombinant 3ABC (r3ABC) was obtained as an insoluble precipitated protein. Although we have used different expression vectors, host *E. coli* strains, incubation temperatures, and times, as well as IPTG concentrations, a minor change in the level of recombinant protein expression was observed as these factors were modified. Our bacterially expressed r3ABC was a fully insoluble protein and not just a membrane associated as nearly all its quantity has been

always precipitated in the pellet fractions. Exceptionally, a trivial amount was gained from these purifications, which was not visualized on SDS-PAGE analysis. However, it was detectable by western blotting analysis. This low yield plus insolubility status of the r3ABC expression could be attributed to the presence of the 3C protein, as its protease activity might result in proteolytic degradation and affect the whole r3ABC protein solubility [14]. Also, after the synthesis of the FMDV-3ABC, it is rapidly degraded and associated with a detergent-insoluble fraction in FMDV infected cells [35].

A previous study reported similar failure to express the native full-length 3ABC polyprotein in prokaryotic (*E. coli*) and baculovirus systems; however, it was possible after inserting point mutations to substitute two out of three active protease sites within the 3C [21]. They were refereed 3C effect to its possession of cysteine protease (chymotrypsin) that may completely cleave the expressed protein [17]. This catalytic activity was at the 163 position of cysteine residue, the 46 position of histidine residue, and at the 84 position of asparagine/glutamine residue [29]. They expected that the substitutions of these amino acids probably deactivated the catalytic 3C effect and suppressing the proteolytic activity and resulting in the high protein yield. Similarly, other researchers [21] substituted the cysteine at 142 and cysteine at 163 positions to serine and glycine, respectively, to inactivate the 3C proteolytic activity that led to the successful prokaryotic expression of the 3ABC polyprotein. Even in these cases, the mutated 3ABC was produced in a denatured status and subjected to dialysis for refolding.

In addition to its protease activity, our data have exposed the hydrophobic properties of the 3C coding region using the computational analysis. The presence of hydrophobic regions in a recombinant protein often raised a toxic effect on the host cells. This is causing the association or incorporation of such a protein into vital membrane systems and resulting in its insoluble form. In contrary to 3C, our investigations revealed that the 3AB region has complete hydrophilic properties; and the omission of the 3C encoding sequence would not affect the 3D structure of the 3AB protein. Thus, and unlike most recombinant proteins, the r3AB was remarkably expressed in a complete soluble product hence the native purification was adequate for its complete recovery and protein folding procedures were unnecessary. Noticeably, the use of extensive imidazole washings allowed the elution of 6xHis-r3AB fractions free from other proteins, with no need to further SDS-PAGE purification or electroelution for any further application of the purified r3AB. The complete purification recovery of r3AB has been measured on an approximate frequent basis by 4 mg per each liter of culture medium.

Interestingly, the obtained results showed that removal of the 3C did not affect the antigenic properties of the purified r3AB comparable to the r3ABC. Immunoreactivity of the

r3AB when it was used as a coating antigen in an indirect ELISA, exhibited unique positive reactivity to anti-FMDV Ab in sera from animals infected with the three different FMDV serotypes (A, O, and SAT 2), confirming its authentic immunoreactivity. It could be explained as the 3AB encoding sequence is highly conserved among the different FMDV serotypes as previously reported [1], the homology percentage of 3AB amino acid sequence is >90 among all FMDV serotypes. As our previously developed recombinant VP2 protein was effectively used earlier for FMDV Abs detection, it was used in parallel with r3AB as a coating antigen. The purified r3AB immunogenicity was demonstrated by its ability to induce anti-3AB Ab in mice. ELISA results revealed a reasonable seroconversion in immunized mice sera with a significant sequential increase in reactivity to the sera collected post r3AB immunization. These results confirmed the potential antigenicity of r3AB protein. This came in harmony with the data generated by computational analysis regarding the 3C removal that has not affected the antigenic properties of 3AB.

Unlike a previous study that described the preliminary 3AB expression yielding insoluble monomers and dimers aggregates [1], the currently generated r3AB was fully soluble monomers and homogenous protein. This difference is attributable to the successful combination of vector-host strain or post-induction growth temperatures used in the respective experiments. The importance and prospective applications of our developed soluble, antigenic, and highly purified r3AB come forward even in FMD-free countries in which FMDV control involves a test and slaughter policy with movement restrictions. Also, getting rid of infected and contact animals may not be enough to eliminate the infection and emergency vaccination may be converted into an additional option.

In conclusion, 3C removal restored efficacy and improved properties of the r3ABC, overcoming its insolubility and degradation, producing a fully soluble recombinant antigen (r3AB). That would be a potential candidate for individuals or herd screening of animals at import-export stations to detect seroconversion to any FMDV serotypes, and to further distinguish between infected and vaccinated animals using a single-step assay. Prospective studies for evaluating the generated r3AB in large number of FMDV infected serum samples which belong to different serotypes are required.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethics statement: Animals used in this investigation have been handled according to the National Institute of Health (NIH) animal care principles and policies. The Animal Care and Use Committee in Veterinary Medicine Cairo University has endorsed all experiments (Vet CU16072020180). This study does not involve any experiments in human conducted by any of the authors.

References

1. He C, Wang H, Wei H, Yan Y, Zhao T, Hu X, Luo P, Wang L, Yu Y (2010) A recombinant truncated FMDV 3AB protein used to better distinguish between infected and vaccinated cattle. Vaccine 28:3435–3439
2. Doel TR (2003) FMD vaccines. Virus Res 91(1):81–99
3. World Organization for Animal Health (2008) In: Foot and mouth disease. Office International des épizooties (ed) Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees) [electronic resource]. Office International des epizooties, Paris
4. Wang JF, Guo YS, Christakos G et al (2011) Hand, foot and mouth disease: spatiotemporal transmission and climate. Int J Health Geogr 10:25
5. Sharma GK, Mohapatra JK, Mahajan S, Matura R, Subramaniam S, Pattnaik B (2014) Comparative evaluation of non-structural protein-antibody detecting ELISAs for foot-and-mouth disease sero-surveillance under intensive vaccination. J Virol Methods 207:22–28
6. Clavijo A, Wright P, Kitching P (2004) Development in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. Vet J 167:9–22
7. Sorensen KJ, Madsen KG, Madsen ES, Salt JS, Nqindi J, Mackay KD (1998) Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Arch Virol 143(8):1461–1476
8. Berger HG, Straub OC, Ahl R, Tesar M, Marquardt O (1990) Identification of foot-and-mouth disease virus replication in vaccinated cattle by antibodies to non-structural virus proteins. Vaccine 8:213–216
9. Lubroth J, Brown F (1995) Identification of native foot-and-mouth disease virus non-structural protein 2C as a serological indicator to differentiate infected from vaccinated livestock. Res Vet Sci 59:70–78
10. Cowan KM, Graves JH (1996) A third antigenic component associated with foot-and-mouth disease infection. Virology 30:528–540
11. Mackay DK, Forsyth MA, Davies PR, Berlinzani A, Belsham GJ, Flint M, Ryan MD (1998a) Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine 16:446–459
12. Rodriguez A, Nunez JI, Nolasco G, Ponz F, Sobrino F, DeBlas C (1994) Direct PCR detection of foot-and-mouth disease virus. J Virol Methods 47:345–349
13. Sorensen KJ, de Stricker K, Dyrting KC, Grazioli S, Haas B (2005) Differentiation of foot-and-mouth disease virus infected animals from vaccinated animals using a blocking ELISA based on baculovirus expressed FMDV 3ABC antigen and a 3ABC monoclonal antibody. Arch Virol 150:805–814
14. Capozzo AVE, Burke DJ, Fox JW, Bergmann IE, La Torre JL, Grigera PR (2002) Expression of foot-and-mouth disease virus

non-structural polypeptide 3ABC induces histone H3 cleavage in BHK21 cells. Virus Res 90:91–99

15. Clarke BE, Sangar DV (1988) Processing and assembly of foot-and-mouth disease virus proteins using subgenomic RNA. J Gen Virol 69:2313–2325

16. Vakharia VN, Devaney MA, Moore DM, Dunn JJ, Grubman MJ (1987) Proteolytic processing of foot-and-mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. J Virol 61:3199–3207

17. Curry S, Roque-Rosell N, Sweeney TR, Zunszain PA, Leatherbarrow RJ (2007) Structural analysis of foot-and-mouth disease virus 3C protease: a viable target for antiviral drugs? Biochem Soc Trans 35:594–598

18. Hartley DL, Kane JF (1988) Properties of inclusion bodies from recombinant *Escherichia coli*. Biochem Soc Trans 16:101–102

19. Carrio MM, Villaverde A (2002) Construction and deconstruction of bacterial inclusion bodies. J Biotechnol 96:3–12. https://doi.org/10.1016/S0168-1656(02)00032-9

20. Carrio MM, Villaverde A (2001) Proteinaggregationasbacterial-inclusion bodies isreversible. FEBS Lett 489:29–33. https://doi.org/10.1016/S0014-5793(01)02073-7

21. Sariya L, Thangthumniyom N, Wajjawalku W, Chumsing W, Ramasoota P, Lekcharoensuk P (2011) Expression of foot and mouth disease virus non-structural polyprotein 3ABC with inactive 3C(pro) in Escherichia coli. Protein Expr Purif 80:17–21

22. Salem R, El-Kholy AA, Ibrahim M (2019) Eight novel single chain antibody fragments recognising VP2 of foot-and-mouth disease virus serotypes A, O, and SAT 2. Virology 533:145–154. https://doi.org/10.1016/j.virol.2019.05.012

23. El-Gaied L, Salem R, Elmenofy W (2017) Expression of tomato yellow leaf curl virus coat protein using baculovirus expression system and evaluation of its utility as a viralantigen. 3 Biotech 7:269. https://doi.org/10.1007/s13205-017-0893-4

24. Elmenofy W, Mohamed I, El-Gaied L, Salem R, Osman G, Ibrahim M (2020) Expression of 1B capsid protein of foot-and-mouth disease virus (FMDV) using baculovirus expression system and its validation in detecting SAT 2- specific antisera. PeerJ 8:e8946. https://doi.org/10.7717/peerj.8946

25. Salem R, Arif AI, Salama M, Osman GH (2018) Polyclonal antibodies against the recombinantly expressed coat protein of the Citrus psorosis virus. Saudi J Biol Sci 25:733–738. https://doi.org/10.1016/j.sjbs.2017.10.018

26. Salem R, Assem KS, Omar AO, Khalil AA, Basry AM, Waly RF, Samir N, El-KholyAA (2020) Expressing the immunodominant projection domain of infectious bursal disease virus fused to the fragment crystallizable of chicken IgY in yellowmaize for a prospective edible vaccine. Mol Immunol 118C:132–141. https://doi.org/10.1016/j.molimm.2019.12.015

27. Salem R, El-Kholy AA, Omar OA, Abu el naga MN, Ibrahim M, Osman G (2019) Construction, expression and evaluation of recombinant VP2 protein for serotype independent detection of FMDV seropositive animals in Egypt. Sci Rep 9:10135. https://doi.org/10.1038/s41598-019-46596-9

28. De Diego M, Brocchi E, Mackay D, De Simone F (1997) The nonstructural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch Virol 142:2021–2033

29. Grubman MJ, Zellner M, Bablanian G, Mason PW, Piccone ME (1995) Identification of the active-site residues of the 3C proteinase of foot and mouth disease virus. Virology 213:581–589

30. Gleeson LJ (2002) A review of the status of foot and mouth disease in South-East Asia and approaches to control and eradication. Rev Sci Tech Off Int Epizoot 21:465–475

31. Yakovleva AS, Shcherbakov AV, Kan'shina AV, Mudrak NS, Fomina TA (2006) Use of the recombinant nonstructural 3A, 3B, and 3AB proteins of foot-and-mouth disease virus in indirect ELISA for differentiation of vaccinated and infected cattle. Mol Biol 40:146–151

32. Lee F, Jong MH, Yang DW (2006) Presence of antibodies to nonstructural proteins of foot-and-mouth disease virus in repeatedly vaccinated cattle. Vet Microbiol 115:14–20

33. Sumption K, Rweyemamu M, Wint W (2008) Incidence and distribution of foot-and-mouth disease in Asia, Africa and South America; combining expert opinion, official disease information and livestock populations to assist risk assessment. Transbound Emerg Dis 55:5–13

34. Lu Z, Cao Y, Guo J, Qi S, Li D, Zhang Q, Ma J, Chang H, Liu Z, Liu X, Xie Q (2007) Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. Vet Microbiol 125:157–169

35. Grigera PR, Sagedahl A (1986) Cytoskeletal association of an aphthovirus-induced polypeptide derived from P3ABC region of the viral polyprotein. Virology 154:369–380