

# Serotyping of foot-and-mouth disease virus by antigen capture-ELISA using monoclonal antibodies and chicken IgY

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## SUMMARY

Laboratory detection of specific foot-and-mouth disease virus (FMDV) is routinely carried out by ELISA and RT-PCR. Identification and serotyping of FMDV by ELISA requires polyclonal antibodies raised in rabbits and guinea pigs. The polyclonal antibodies have certain disadvantages such as batch to batch variation, inconsistent yields of antibodies and limited quantity of serum obtained from individual animals. This paper describes a method wherein monoclonal antibodies and chicken IgY were used in an antigen capture-ELISA for serotyping of thirty tongue epithelial samples and sixty tissue culture fluids. The results were compared with the routine antigen detection ELISA. The present study indicated that monoclonal antibodies and chicken IgY can substitute conventional polyclonal antibodies for routine serotyping of FMDV.

**KEY WORDS:** Foot-and-mouth disease, FMDV, Serotyping, ELISA, IgY

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Laboratory diagnosis of foot-and-mouth disease (FMD) is carried out by detection of specific foot-and-mouth disease virus (FMDV) in epithelial tissue suspensions and tissue culture supernatants using ELISA and RT-PCR with different modifications (Reid *et al.*, 2003).

ELISA based identification and serotyping of FMDV employs rabbit and guinea pig polyclonal antibodies (PAb). Production of PAb involves appropriate care and maintenance of the laboratory animals, which requires the approval by the animal ethics Committee. The use of PAb has

certain limitations such as batch to batch variation, inconsistent yields of antibodies and scanty serum obtained from individual animals. The IgY derived from hyperimmune chicken egg yolk has been recognized as an excellent, alternative source of PAb (Hodek and Stiborova, 2003, Warr *et al.*, 1995, Polson and Von Wechmar, 1980 and Jensenius *et al.*, 1981).

There is minimal or no cross-reaction with mammalian IgG (Ambrosius and Hadge, 1987 and Larsson and Sjoquist, 1990), complete absence of non-specific binding and elimination of the need for cross-species immuno absorptions (Larsson *et al.*, 1993) due to the phylogenetic distance between chicken and mammals. We have developed an antigen capture-ELISA for serotyping of FMDV utilizing the chicken IgY and monoclonal antibodies (Mab). The antigen capture-ELISA reported in this paper offers a potential substitute for the existing ELISA wherein polyclonal serum is used.

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Tongue epithelium (n=30), collected from FMD suspected animals, as a 10% (w/v) suspension in 0.04M phosphate buffer and tissue culture adapted FMDV serotypes O, A and Asia1 (n=60) available in our laboratory were used to develop an antigen capture-ELISA format using IgY and Mabs. In addition, RT-PCR typing (Reid *et al.*, 1999) was carried out on all the samples to confirm the serotype of FMDV.

White leghorn layer hens of 20 weeks age, weighing 1.75-2.00 kg body weight were used for immunization with FMDV antigen. The hens were provided with *ad libitum* feed and water and maintained in separate layer cages as per the approved requirements.

Vaccine strains currently in use in India viz. O IND R2/75, A IND 17/82 and Asia1 IND 63/72 were grown in BHK-21-CZ suspension cells. The clarified supernatant obtained 48 hours post infection was inactivated with two doses of binary ethyleneimine [BEI] (Aarthi *et al.*, 2004). Inactivation kinetics, virus amplification tests and inocuity tests were conducted to ensure the safety and inocuity of the antigens.

The inactivated virus was purified using a linear sucrose density gradient (Doel *et al.*, 1982) and quantified using a spectrophotometer (Doel and Mowat, 1985). Purified 146S antigens were stored in aliquots in liquid Nitrogen at -196°C, till use. Hens were inoculated intramuscularly with 50µg of the inactivated FMDV 146S antigen emulsified with equal volume of Freund's complete adjuvant. Two inoculations at an interval of 2 weeks were given on day 0 and day 14. Eggs were collected daily from day 14 post immunization. The yolk was separated and stored at -20°C until further processing.

For small-scale purification of IgY from egg yolk a commercial kit, *EggsPure* IgY kit (AGRO-BIO™, St. Aubin, France) was used. The purity of the antibodies was checked by both 10% reducing and non-reducing SDS-PAGE. The antibodies were stored at -20°C till use. An in-house method for large scale purification of egg yolk IgY was also developed and used in this study. Egg yolk was collected on a Whatman filter paper No.1 (Whatman, USA), after carefully discarding the egg albumin. The egg yolk (10ml) was mixed with 40 ml of phosphate buffered saline (pH 7.6). Then 1.75 g of polyethylene glycol (PEG pulverized, MW 6000, Sigma, USA) was added to achieve a fi-

nal concentration of 3.5% (w/v). The mixture was stirred at 24±2°C for 30 minutes and centrifuged at 12000 x g for 30 minutes. The supernatant was filtered through a Whatman filter paper No.1 (Whatman, USA). PEG was added to the filtrate to achieve a final concentration of 12% (w/v). The mixture was stirred at 24±2°C for 30 minutes and then centrifuged at 12000 x g for 10 minutes.

The supernatant was discarded and the pellet was dissolved in 2.5 ml of phosphate buffered saline (pH 7.2; without sodium chloride). The mixture was allowed to stand at 24±2°C overnight. The next day an equal volume of saturated ammonium sulfate solution (4M) was added to the mixture and the contents were stirred at room temperature (25°C) for 30 minutes. The mixture was centrifuged at 12000 x g for 10 minutes and the pellet was dissolved in 2.5 ml of phosphate buffer solution (pH 7.2; without sodium chloride). The mixture was dialyzed overnight against phosphate buffer solution (pH 7.2; without sodium chloride). Antibodies were further concentrated over dry PEG (MW 6000) for 30 minutes.

The protein content was measured calorimetrically. The purity of the antibodies was checked by both 10% reducing and non-reducing SDS-PAGE. Analysis of chicken egg yolk derived IgY by 10% SDS-PAGE electrophoresis (non-reducing and reducing) by the kit method and by the in-house method is given in Figures 1 and 2.

The protein estimation was done using the bicinchoninic acid kit (Sigma, USA) which is similar to the Lowry procedure (Table 1). This kit was a microtiter plate method wherein standard protein in a linear concentration range is used to extrapolate the test sample's protein concentration. Serial twofold dilutions of the standard protein (BSA) and sample were done in a microtiter plate followed by the addition of the kit reagents as per the manufacturer's recommendations. After incubation at 37°C for 30 minutes, the color intensity was read at A<sub>562</sub> nm. A dot scatter plot of the standard protein was plotted with the absorbance values along the Y-axis and the protein concentrations along the X-axis. An R<sup>2</sup> value of ≥99 was considered as satisfactory for the graph. A similar dot plot was made for the sample. The slope value of the standard was equated with the dilution of the standard from which the graph was plotted. Hence the protein estimate of the sample was cross-calculated after including the sample

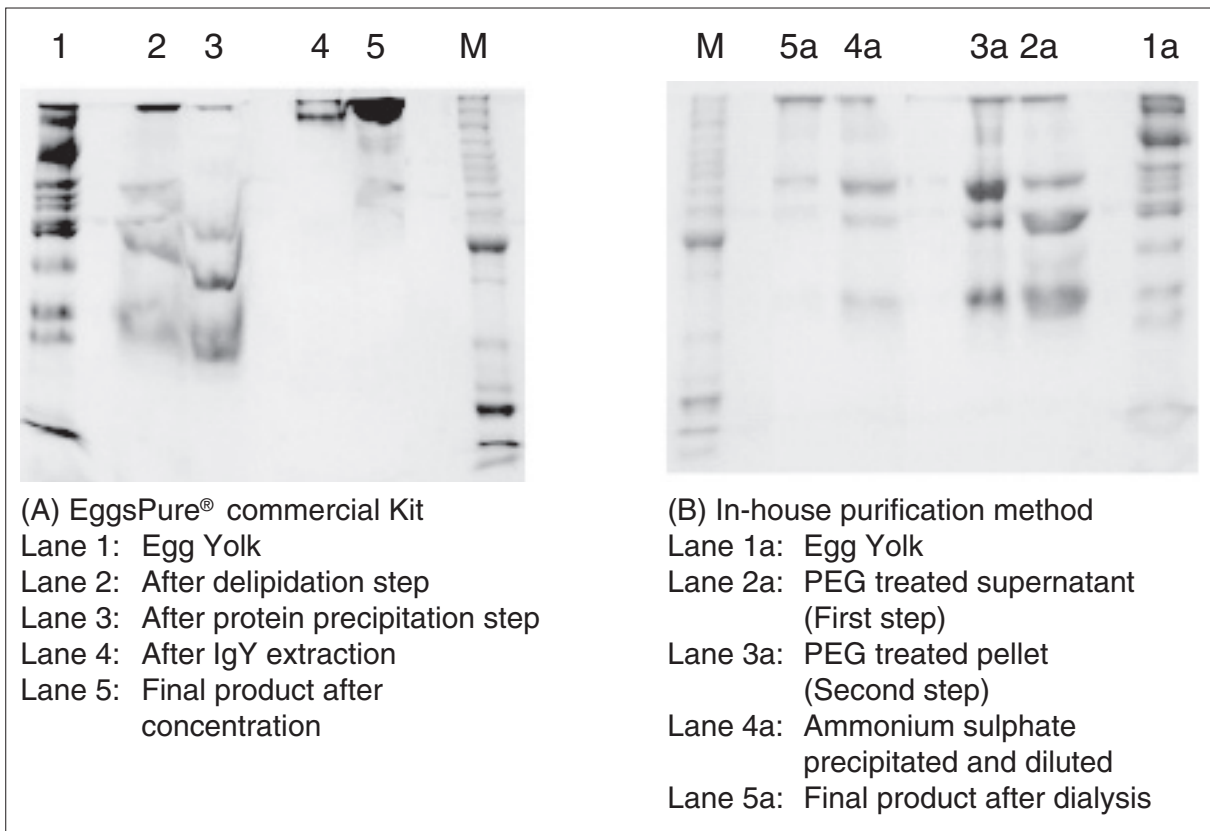


FIGURE 1 - Analysis of purification steps of chicken egg yolk derived IgY by 10% SDS-PAGE Electrophoresis (Non-reducing) (A) EggsPure® commercial Kit (Lane 1, 2, 3, 4 and 5) and (B) In-house purification method (Lane 1a, 2a, 3a, 4a and 5a); M denotes Benchmark® protein molecular weight marker.

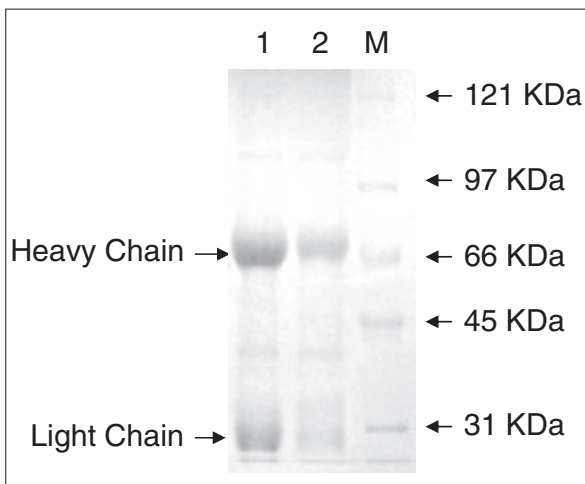


FIGURE 2 - Analysis of purified chicken egg yolk derived IgY by 10% SDS-PAGE Electrophoresis (reducing) Lane 1: EggsPure® commercial Kit and Lane 2: In-house purification method; M denotes protein molecular weight marker (MBI Fermantas).

dilution factor and expressed in units as that of the standard. The antibodies were stored at -20°C until further use.

Monoclonal antibodies (Mabs) were prepared using standard protocols. Briefly four 6 to 8 weeks old female Balb/c mice were immunized with BEI inactivated, purified, 146S antigen of all the three FMDV serotypes viz. O TNN 24/84, Asia 1 WBN 117/85, and A IND 17/82. One week after a final booster inoculation, the spleen was harvested from the mice and the splenocytes were fused with murine myeloma cells using standard protocols.

Polyclonal hybrids were selected based on their reactivity with the respective 146S antigens in a sandwich ELISA (Hamblin *et al.*, 1984). The monoclonal hybrids were selected after two rounds of single cell cloning. The monoclonals were propagated and checked for FMDV serotype specific Mab secretion using the sandwich ELISA as men-

TABLE 1 - Comparative IgY yields from egg yolk using EggsPure® kit and in-house IgY purification method.

EggsPure® Kit	mg/ml	In-house method	mg/ml
Egg yolk	26.84	Egg yolk	4.82
After delipidation step	1.38	PEF treated supernatant (first step)	4.45
After protein precipitation step	0.31	PEG treated pellet (second step)	69.06
After IgY extraction	0.25	Ammonium sulphate precipitated and diluted	5.74
Final product after concentration	1.25	Final product after dialysis	19.16

tioned above. Three MAbs viz., OD3, 1G6 and A19F9 specific to FMDV serotypes "O", "Asia 1" and "A", respectively were chosen for the development of antigen capture-ELISA after characterization (Data not shown).

The MAbs and IgY developed against the three FMDV serotypes were checked for sensitivity and specificity separately by checker board titration of antigen and antibody. The titration was performed using the sandwich ELISA format wherein the serotype specific immune rabbit PABs were used to capture the specific antigen to arrive at an optimal working dilution.

Serotype specific MAbs appropriately diluted in carbonate-bicarbonate coating buffer (pH-9.6) were coated onto ELISA plates (Nunclon®, USA). The plates were incubated in a moist chamber overnight at 4°C. The next day the test antigens (10% (w/v) suspension of tongue epithelia (TE) and the BHK-21 adapted virus) were added to the

wells and incubated for an hour at 37°C. The plates were washed and peroxidase conjugated serotype specific IgY appropriately diluted in a blocking cum diluting buffer containing phosphate buffered saline with 0.05% Tween 20 and 10% normal bovine serum was added as detecting antibodies. The plates were incubated for an hour at 37°C.

The plates were washed and substrate and chromogen mix (hydrogen peroxide/o-phenylenediamine hydrochloride) was added. The color development was stopped with 1.25M sulfuric acid after 15 minutes of incubation at room temperature in a dark chamber. The absorbance was read at 492 nm wavelength using an ELISA reader (Molecular Devices, USA). The results of the different ELISA formats were compared with the routine antigen detection ELISA performed in the laboratory (Hamblin *et al.*, 1984).

The comparative results of FMDV serotyping of

TABLE 2 - Comparative results of FMDV serotyping using routine antigen ELISA, serotyping RT-PCR and new ELISA using Monoclonal antibodies and Chicken IgY.

Type of Sample	Virus Serotype	Virus serotyping		
		Routine ELISA*	RT-PCR*	New ELISA* (Mabs +IgY)
Tongue Epithelium (n=30)	Type O	5 (16.67)	8 (26.67)	8 (26.67)
	Type A	0 (0)	3 (10)	2 (6.67)
	Type Asia1	0 (0)	1 (3.33)	1 (3.33)
	No Virus Detected	25 (83.33)	18 (60.33)	19 (63.33)
Tissue Culture Fluid (n=60)	Type O	23 (38.33)	24 (40.00)	24 (40.00)
	Type A	13 (21.67)	13 (21.67)	13 (21.67)
	Type Asia1	21 (40)	21 (40)	21 (40)
	No Virus Detected	3 (5.00)	2 (3.33)	2 (3.33)

\*Values in parenthesis indicate (%) percentage positive samples.

10% (w/v) TE suspension (n=30) and tissue BHK-21 adapted FMDV belonging to serotypes O, A and Asia1 (TCF; n=60) are shown in Table 2. Twelve out of thirty TE samples could be directly serotyped using Mab IgY based ELISA whereas only seven could be serotyped using the regular antigen detection ELISA. All the samples serotyped by Mab-IgY ELISA were confirmed by RT-PCR except for one serotype A sample which could not be detected using the Mab-IgY ELISA. Eighty eight out of 90 samples could be serotyped using Mab IgY ELISA and the routine antigen ELISA. The Mab-IgY ELISA showed 100% specificity with the RT-PCR results while the sensitivity was around 98.87%.

All 32 serotype O viruses were identified as FMDV serotype O by the antigen capture-ELISA using Mabs and chicken IgY. Fifteen serotype A viruses could be detected by the Mab-IgY ELISA while 13 out of 16 serotype A viruses could be identified by the routine method (Hamblin *et al.*, 1984). All 22 serotype Asia1 isolates could be identified by the new ELISA whereas only 21 could be serotyped as Asia1 by the routine method.

The results of Mab-IgY ELISA and RT-PCR were comparable except for one serotype A sample. High specificity and comparable sensitivity with RT-PCR indicated that use of Mabs and chicken IgY antibodies would be a viable alternative for polyclonal sera raised in laboratory animals like rabbits and guinea pigs. Two methods were used for purification of IgY and both the methods gave similar yields of total IgY.

On an average around 5-6 mg of antibodies could be purified from yolk separated from a single egg. For each antigen approximately 900-1500 mg of IgY antibodies per bird can be produced. Considering the specificity and sensitivity of these antibodies against specific antigens the working dilution can be anywhere around 1:2000 to 1:4000 when used as detection antibodies. The yolk that is separated and stored at -20°C is stable for long duration and the IgY can be purified whenever required.

It is needless to emphasize the usefulness of Mabs in achieving sufficient sensitivity and specificity of the ELISA. The Mabs used here are reagents of unlimited supply, unique specificity and excellent sensitivity and so far as has been shown, react only with the respective antigens against which they are raised (Smitsaart *et al.*, 1990). The pro-

duction of Mabs can be optimized and easily scaled up using recent technologies of cell culture involving either wave bioreactor or fibrastage system.

The purification protocols for Mabs have been well documented to obtain maximum yields from each batch. With the availability of both Mabs and IgY against specific antigens, sensitive ELISA procedures can be developed without the problems of batch variation and cross-reactivity. The present study indicated that Mabs and IgY can replace the conventional polyclonal antibodies for detection of FMDV antigens in routine immunoassays.

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