

# Monoclonal Antibodies Specific to Water Buffalo (*Bubalus bubalis*) Myxovirus Resistance Protein<sub>1</sub>

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**Abstract:** Recombinant bubaline (*Bubalus bubalis*) myxovirus resistance protein 1 (bbMx1) was successfully expressed by an *Escherichia coli* expression system. After immunization and cell fusion, a set of ten mouse hybridomas producing mAbs to bbMx1 was established. The ten corresponding mAbs were further characterized using indirect ELISA, western blot analysis and immunocytofluorescent staining. Eight mAbs, designated 11C7, RD5, NF3, 9D1, FD4, PE6, 11A7 and 10F5, displayed binding abilities and specificity in the three formats. Moreover, combining NF3 (for capture) with RD5 (for detection), 9D1 with RD5, FD4 with 11C7 or PE6 with 11C7 generated a strong signal in a prototype sandwich-ELISA. The results suggest that the mAbs developed and characterized here provide an excellent starting point for developing diagnostic tools aimed at detecting viral infections in the water buffalo, whatever using immunoblotting, immunocytofluorescence or sandwich-ELISA.

**Keywords:** Water buffalo, MX, antibody, biomarker, viral infection.

## INTRODUCTION

The everyday movement of animals between farms and countries is an unavoidable reality of our globalized world. Although these animals generally appear clinically healthy at the place of departure, the probability that a fraction of the transported population carry a transmissible viral infection that becomes clinically noticeable only upon arrival is non-zero. Further, in the context of an ongoing animal health crisis, large cohorts of animals suspected of being infected by a highly transmissible virus are systematically slaughtered. Post hoc virological tests conducted in the past in such circumstances showed that the vast majority of these animals were not actually infected. Unfortunately, due to the lack of reliable ante-mortem tests to know if an animal is infected or not, the use of such massive, financially heavy and bioethically unacceptable slaughter remains essential for crisis management. Finally, because the diagnosis can only be made on the basis of assumptions made a priori, it is financially unrealistic to test animals suspected or to be transported for each significant viral disease. Thus, effective management of contagious animal diseases would dramatically benefit of a cheap, high-throughput-compatible tool that can detect a viral infection as soon

as possible after the infection and whatever the causative virus, including if it is a new, emerging virus.

In all vertebrates, sensing of any viral infection leads to a universal response: the secretion of type I/III interferons, which contributes to host defence by establishing an antiviral state in target cells [1-3]. Viruses induce predominantly three classes of IFNs, namely IFN $\alpha$ , - $\beta$  and - $\lambda$ , collectively called type I/III IFNs. Binding of these IFNs to their receptors induces transcription of many IFN-stimulated genes among which Mx genes are of particular interest. It has been proven difficult to detect circulating IFNs in the blood of patients with viral infections because of their short biochemical and biological half-lives hence hindering their use as biomarkers of viral infection [1, 4]. On the opposite, mammalian Mx GTPases appear as promising biomarkers for such infections as their expression is known to be strictly subordinated to auto- or paracrine cell stimulation by type I/III interferons. This specificity arises from the presence in the promoters of Mx genes of a particular sequence called "interferon-sensitive regulatory element" which is repeated in tandem [5-7].

Furthermore, *in vivo*, Mx proteins appear within 1-2 h of type I/III stimulation, peak within 36 h and their half-life extends over 48 h [4], [8-10]. The usefulness of Mx proteins as biomarkers has been duly confirmed in a series of pediatric studies aimed at discriminating viral from bacterial infections in young children [11-15].

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Further, human Mx1 protein has been used as a biomarker to prove local multiplication of a live attenuated vaccine [16] and is currently used to confirm the efficacy of interferon therapy in HCV-infected people [17] and to monitor IFN- $\beta$  therapy in multiple sclerosis patients [18].

Water buffalo (*Bubalus bubalis*) has been an integral part of livestock agriculture in Asia for over 5000 years, producing draft power, milk, meat and hides [19]. Today, more than 200 million buffaloes provide 120353705 tons of milk and 3838647 tons of meat and, in several countries, up to 30% of the draft power for agricultural operations [20, 21]. Unfortunately, the species did not receive the attention of policy and diagnostic tool makers in accordance with its socio-economical merits. To develop a clinically significant, cheap and practical enzyme-linked immunosorbent assay (ELISA) for the detection and quantitation of Mx1 protein in water buffalo whole blood, specific ligands are crucially needed.

The work presented in this paper aims to provide key ligands needed for studying buffalo's Mx1 biology. We hence successfully expressed recombinant water buffalo Mx1 in *Escherichia coli* which enabled us to generate and characterize a set of 10 monoclonal antibodies (mAb). These mAb can be used in immunoblotting or immunofluorescence experiments, as well as to develop an ELISA tool specifically dedicated to the quantitation of this biological marker of viral infections.

## MATERIALS AND METHODS

### Construction of Mx1 Protein Expression Vectors

Two vectors were synthesized for expressing a reference buffalo Mx1 (NP\_001277782, SM1) either in *Escherichia coli* (named pET-28b[+]/His<sub>6x</sub>-bbMx1, SM2) or in mammalian cells (pD657RA/V5-bbMx1). In both cases, the recombinant water buffalo Mx1 intended was fused to an N-term functional group, either a poly-histidine tract (pET-28b[+]-His<sub>6</sub>-bbMx1) or a V5 epitope (pDA657RA/V5-bbMx1). In the latter, the V5-bbMx1 protein was further fused to an N-term bipartite group consisting of the fluorescent marker protein RFP followed by the spontaneously cleavable peptide 2A. In both cases, the CDS expected to encode the recombinant protein aimed was codon-optimized, artificially synthesized by solid-phase DNA synthesis, and cloned in the said vectors. Once the capacity of pDA657RA/V5-bbMx1 to drive appropriate expression

of V5-bbMx1 in HEK-293T cells was duly checked, similar plasmids expressing V5 epitope-flanked porcine (NP\_999226) and equine (NP\_001075961) Mx1 were synthesized. The sequences of the inserts of interest were verified by sequencing before use.

### Purification of Recombinant His<sub>6x</sub>-bbMx1 Protein in Prokaryotic Cells

The recombinant pET-28b[+]/His<sub>6x</sub>-bbMx1 (Novagen) prokaryotic expression vector was used to transform competent *E. coli* Rosetta (DE3) pLysS cells (Novagen), which were cultured at 37°C for 4h in 200 mL of LB medium containing kanamycin, followed by the addition of 1 mM isopropylthio- $\beta$ -D-galactoside for 2h and then harvested by centrifugation (1,000 g for 10 min at 4°C). The pellets were washed with phosphate-buffered saline (PBS), then stored at -80°C. Total protein extraction and purification under denaturing conditions were done with, and as recommended for the ProBond™ purification system (Invitrogen). One microgram of whole protein extracts from pooled elution fractions showing a measurable absorbance at 280 nm was supplemented with 0.5% Triton-X100, dialyzed stepwise against successive dilutions of PBS-urea and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions showing a clear and unique band at ~75 kDa on the gels were then pooled, supplemented with 0.5% Triton-X100, and dialyzed stepwise against successive dilutions of PBS-urea (6–0.5 M). The concentration of recombinant His<sub>6x</sub>-bbMx1 in the resulting stock solution was determined with the Pierce™ BCA Protein Assay Kit. The stock solution of recombinant His<sub>6</sub>-bbMx1 was used for the hyperimmunization campaign and for the direct ELISA aimed at screening the collection of hybridomas' supernatants.

### Expression of Recombinant V5-bbMx1 Protein in Mammalian Cells

Pure suspensions of mammalian cells expressing V5-bbMx1 were produced to characterize the expected anti-Mx1 mAbs in immunoblot and immunofluorescence formats. Thawed HEK-293T cells (ATCC® CRL-1573™) were first passaged thrice in DMEM (with 10% FCS, 1% pen-strep and 0.5% amphotericin B) and then transfected with pDA657RA/V5-bbMx1 by use of the Lipofectamine-3000 Transfection reagent kit (ref.#L3000-008, ThermoFisher) according to the manufacturer's instructions. Briefly, for transfection of an 80-90% confluence monolayer in a T75 cell culture

flask, an amount of 24 µg of the plasmid DNA was diluted in P300 reagent (dilution 1:2 w/v), the mix is then added in the Lipofectamine 3000 reagent diluted in Opti-MEM medium (dilution 1:16 v/v). The final mixture was added dropwise into the T75 flask, and the latter was reincubated at 37°C for 48h in a 5% CO<sub>2</sub> and 80% RH atmosphere. RFP-positive cells were then sorted out by flow cytometry and were used for subsequent mAb characterization studies. A similar protocol was followed to express the two other mammalian Mx1 proteins.

### Hyperimmunization, Cell Fusion, and Cloning

Five healthy 8 weeks old female BALB/c mice were immunized with the recombinant V5-bbMx1 protein. The monoclonal hybridoma cells were made by fusion with spleen cells derived from the immunized mice and SP2/0 myeloma cells at a ratio of 5/1, and fusion was carried out with 50% PEG-400. The hybridoma cells were resuspended in RPMI 1640 medium supplemented with 20% fetal calf serum, HAT, and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). Fusion products were seeded in 96-well plates containing the feeder cells and cultured in a CO<sub>2</sub> incubator. The supernatants of hybridomas were screened by indirect ELISA using plates coated either with the recombinant His<sub>6</sub>-bbMx1 or with a nonpertinent His-tagged protein (for eliminating mAbs targeting the poly-histidine tag). Cells from positive wells were further selected on the basis of the magnitude of the signal-to-noise ratio. To ensure monoclonality, single cells producing a winning antibody were recloned two times by limiting dilution. Stable hybridoma clones were then propagated *in vitro*. Antibodies from monoclonal hybridoma cells were then purified from the supernatants by affinity chromatography using Protein-A Sepharose fast flow (GE Healthcare Europe GmbH) according to the manufacturer's instructions. The subclass of the monoclonal antibody was determined by a commercial mouse mAb isotyping kit (RD-Biotech).

### Indirect ELISA for Anti-Mx1 mAb Detection

Indirect ELISA was performed using the following procedure. Ninety-six well ELISA plates (Costar) were coated with 1.0 mg/ml of purified His<sub>6x</sub>-bbMx1 or nonpertinent His<sub>6x</sub>-protein diluted in carbonate buffer, pH 9.6, in a 100 µl volume and incubated overnight at 5°C. After four washes with PBS containing 0.05% Tween 20 (PBST), either the hyperimmune sera or hybridoma supernatants to be tested were diluted in

PBS containing 5% skimmed milk, 10% horse serum and 3% *E. coli* lysate; 100 µl of the resulting solutions were introduced in each well and were incubated for 30 min at 37°C. After four washes, horseradish peroxidase-conjugated rabbit anti-mouse IgG serum (Sigma) was added to the wells in the same dilution buffer at appropriate working concentration, again 100 µl per well, and incubated at 37°C for 30 min. After four washes, the colour was developed using the chromogen/substrate mixture 3,30,5,50-tetramethylbenzidine/H<sub>2</sub>O<sub>2</sub>. After 15 min, the reaction was stopped by the addition of 2.0 M H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) of each well was read at 450 nm using a microplate reader (Bio-Rad).

### *Bubalus bubalis* Primary Cells Isolation and Culture

Tissue was harvested post-mortem from the core of the suspensory ligament of a five-days old male Asian buffalo calf from a local dairy farm. The calf died from adverse environmental conditions and had no history of systemic infection or other diseases. Pieces of tissue (~50g) were recovered using scalpel and forceps, dipped in 10% iodine polyvidone solution, and washed three times with phosphate-buffered saline (PBS, Lonza) supplemented with 1% penicillin-streptomycin (Gibco) and 1% Amphotericin B (Gibco). Subsequently, tissue samples were cut into small fragments (2-5 mm<sup>3</sup>) under aseptic conditions, washed twice with PBS, and processed as described [22]. Briefly, fragments were digested for 18h at 37°C and 5% CO<sub>2</sub> in a solution containing 0.2 % (w/v) collagenase A (Roche) in PBS supplemented with 1 % penicillin-streptomycin and 1% Amphotericin B. After incubation, the digestion solution was filtered using a 40 µm cell stainer (BD Falcon) and centrifuged at 300 g for 8 min. The cell pellet was washed twice with PBS, resuspended in Dulbecco's modified Eagle's minimal essential medium (DMEM) with 1 g/L glucose and GlutaMAX (Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco), 1 % penicillin-streptomycin and 1 % amphotericin and cultured at 37 °C in a humidified atmosphere containing 5.0 % CO<sub>2</sub>. The medium was changed every three days until cell cultures reached confluence. For passaging, cells were washed with PBS and detached by using 0.05 % trypsin- EDTA (Lonza). Parts of the cells were activated for 24 hours with 1 µg/ml poly(I:C) (InvivoGen).

### Immunoblotting

The ten hybridoma supernatants yielding the best signal-to-noise ratio in the aforementioned indirect

ELISA format were further characterized by immunoblotting. RFP-positive and -negative HEK-293T cell suspensions were washed with PBS at 4°C and pelleted by low-speed centrifugation. Cells pellets were lysed in RIPA buffer and reduced by mixing with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and reducing agent. Subsequently, samples were heated at 70°C for 10min, and 10µL of each were loaded in the well of NuPAGE™ 4-12% Bis-Tris Protein Gels (ThermoFisher Scientific) and run at 200 V for 60 min using the XCell SureLock™ mini-cell electrophoresis system (Invitrogen). Migrated proteins were then transferred onto polyvinylidene difluoride membrane (Thermo Fisher Scientific), nonspecific binding sites were blocked with skim milk (5% in PBST) for 1h at RT and membranes were probed overnight at 4°C with either hyperimmune mouse sera or hybridoma supernatants diluted in skim milk (2,5% in PBST). The blots were then washed in PBST and incubated for 1h at RT with a horseradish peroxidase-conjugated goat pAb raised against mouse IgGs (diluted 1/1000 v/v, Abcam). Finally, the membranes were washed with PBST on a plate shaker for 5 min, then in deionized water for 2 min. Detection of horseradish peroxidase on membranes was made by use of the chemiluminescent substrate Novex® ECL (Invitrogen). Results were visualized using the imaging system ImageQuant™ LAS 4000 and ImageJ software. The membranes were then washed in PBST before being incubated 1h at RT with an HRP-conjugated antibody targeting β -actin (diluted 1/25000 v/v, Abcam). The detection and visualization procedures were done as just described.

The abovementioned experiment was repeated with hybridoma supernatants 11A7 and RD5 and protein extracts from activated versus inactivated *Bubalis bubalis* primary cells.

### Immunocytostaining

The 10 hybridoma supernatants yielding the best signal-to-noise ratio in the aforementioned indirect ELISA format were further characterized by indirect immunofluorescence. RFP-positive and negative HEK-293T cell suspensions were obtained as aforementioned, cytopinned and then fixed by incubating in paraformaldehyde 4% (v/v) at RT for 30 min. The fixed cells were washed with PBST, treated with 0.2% (v/v) Triton X-100 for 5 min to permeabilize the cell membrane, incubated with Dako-Protein Block Serum-free at RT for 2h for blocking nonspecific

binding, and then overnight at 4°C with 10 µg/ml of either an anti-V5 epitope mAb or each of the purified anti-buffalo Mx1 protein mAbs. After washing thrice with PBST, 200 µl of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin antibody diluted 1/200 (v/v) (Abcam) was added onto each cytopin at RT for 60 min in the dark. Excessive reagents were then removed by washing with PBST, and cellular nuclei were stained with DAPI (Aldrich, code no. D9564-10MG). The slides were then thoroughly washed with PBST, mounted with Prolong Gold antifade medium (Thermo Fisher Scientific) and FITC/DAPI signals were examined using a Leica TCS SP5 confocal fluorescence microscope using 488nm and 405 nm laser filters.

### Sandwich ELISA

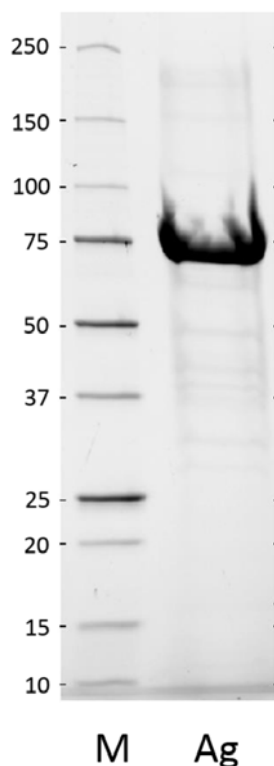
A non-competitive indirect sandwich enzyme-linked immunosorbent assay (ELISA) was developed, using mAbs 11C7, RD5, NF3, 9D1, FD4, PE6, 11A7 or 10F5 for capture, the same biotinylated mAbs followed by HRP-conjugated streptavidin for detection, and finally TMB conversion as the read-out parameter for enzyme activity (Enhanced K-Blue, Neogen). First, 96-well Microlon 600 plates (Greiner) were coated overnight at 4°C with 500 ng/well of said mAbs diluted in carbonate buffer (100 mM, pH 9.5). They were then blocked for 1h at 37°C with 250 µl casein solution (1% in PBS). The biotinylation reagent was prepared by dissolving B (long arm) N-hydroxysuccinimide ester in DMSO. Monoclonal antibodies to be biotinylated were dialyzed against sodium bicarbonate buffer, pH 8.5. The biotinylation reagent (100 to 150 pg ester/mg antibody) was mixed with antibodies (2.0 to 2.5 mg/ml), and the mixture was incubated for 2h at 22°C with occasional stirring. Thereafter, the mixture was dialyzed extensively against sodium bicarbonate buffer to remove the biotinylation reagents. Final dialysis was against PBS. For bbMx1 determinations, the wells were incubated for 1h at 37°C with 100 µl recombinant bbMx1 stock diluted in PBS to the appropriate concentrations (1 or 0,125 µg/ml), then incubated for 1h at 37°C with 100 µl of biotinylated mAbs diluted 1:1000 in PBS-0.5% casein. mAB concentrations (1 or 0,125 µg/ml) were determined by trial and error in order to compensate for the different binding affinities. For detection of immune complexes, the wells were incubated for 1h at 37°C with 100 µl HRP-conjugated streptavidin diluted 1:1000 in PBS-0.5% casein, then incubated at room temperature for 20 min with 100 µl TMB in substrate buffer with H<sub>2</sub>O<sub>2</sub>, according to the

manufacturer's recommendations. Development was stopped by adding 100  $\mu$ l of 1 M HCl, and the plates were read at 450 nm. The OD was determined with respect to a subtractive reference (PBS).

## RESULTS

### Expression of Recombinant Water Buffalo Mx1

The antigenic solution obtained after purification of *E. Coli* protein extract showed a clear and unique band at the expected molecular weight (~75 kDa) on SDS-PAGE gels (Figure 1). The recombinant protein's whole mass was measured by electrospray ionisation mass spectrometry and the measured mass matched that predicted from its sequence. Finally, the nature of the recombinant protein purified was further confirmed by N-terminus amino acid analysis using Edman degradation.

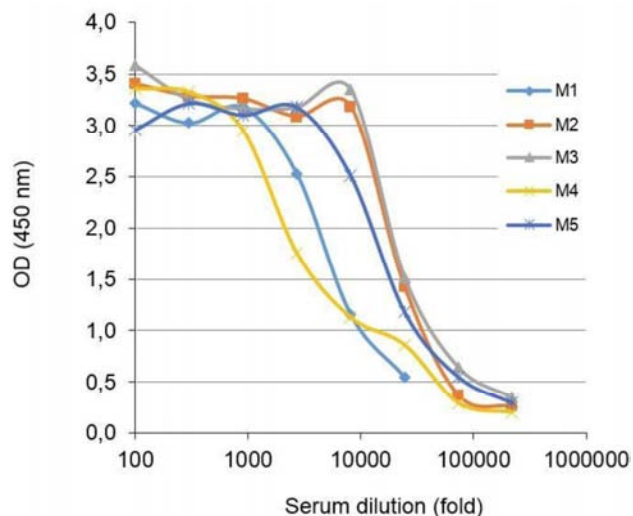


**Figure 1:** SDS-PAGE analysis of the recombinant His<sub>6x</sub>-Mx1 (10  $\mu$ g) prepared for hyperimmunization (reducing condition). Lane M, molecular weight marker (kDa); lane Ag, purified water buffalo Mx1 (1,37 mg/mL). The concentration of recombinant His<sub>6x</sub>-bbMx1 in the antigenic solution was determined with the Pierce™ BCA Protein Assay Kit. See text for key.

### Derivation of Anti-Mx1 Monoclonal Antibodies

All the hyperimmunized mice mounted a strong antibody response against buffalo Mx1 (Figure 2).

Several hundred mouse hybridomas were screened by ELISA for the secretion of antibodies binding either recombinant His<sub>6x</sub>-bbMx1 or a nonpertinent His-tagged protein. A set of 41 hybridomas' supernatants displayed a signal-to-noise ratio greater than 25 (His<sub>6x</sub>-bbMx1 vs. His<sub>6x</sub>-tagged nonpertinent protein). The 15 mAb-producing hybridomas of which the signal-to-noise ratio surpassed 35 were selected for further cloning. Five were not retrieved, and the remaining were subcloned and stored frozen (-20°C). The 10 winning subclones, designated FD4, OB8, PE6, RD5, 7B3, 10B4, 11A7, 10F5, 9D1 and 11C7, were expanded and used to produce the corresponding mAbs. Their viability after thawing was >80%. The 10 purified mAbs were used in all subsequent experiments to detect buffalo Mx1 protein either by ELISA, immunoblotting or immunocytochemistry. In the ELISA format, they always displayed a signal-to-noise ratio above 35.



**Figure 2:** Total antibody response of the 5 mice hyperimmunized against recombinant water buffalo Mx1. Mice #2 and #3 were chosen for hybridoma development. See text for key.

### Characterization of the 10 anti-Mx1 mAbs

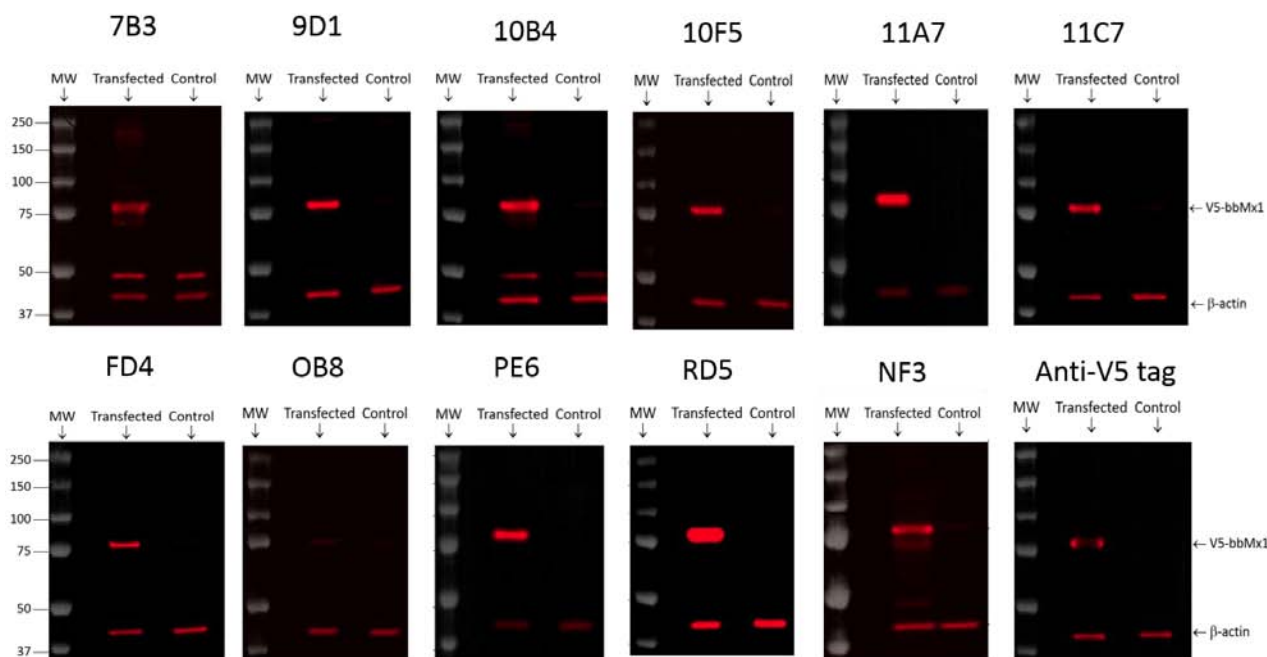
As shown in Table 1, western blot analysis demonstrated that all mAbs detected recombinant V5-bbMx1 in transfected cell extracts (although only a weak signal was obtained with mAb OB8) (Figure 3). Besides, mAbs 7B3 and 10B4 reacted with an additional ~50 kDa band. As the same band was detected in control, non-transfected cells, it is suggested that 7B3 and 10B4 cross-react with a cell protein rather than with a bbMx1 degradation product.

To confirm our results, we repeated the western blotting experiments with the two mAb yielding the

**Table 1: Properties of Anti-Mx1 Monoclonal Antibodies**

mAb	Isotype	Western blotting			Immunocytochemistry	
		RFP-positive cells		Control cells	RFP-positive cells	Control cells
		~75 kDa	~50 kDa			
anti-V5 tag	IgG2a	+	-	-	+	-
FD4	IgG1	++	-	-	++	-
OB8	IgG1	-	-	-	-	-
PE6	IgG1	++	-	-	++	-
RD5	IgG1	+++	-	-	++	-
7B3	IgG2a	+	+	+	++	++
10B4	IgG2a	++	+	+	++	-
11A7	IgG1	++	-	-	++	-
10F5	IgG1	++	-	-	++	-
9D1	IgG1	++	-	-	++	-
11C7	IgG1	++	-	-	++	-

Detection of V5-tagged bubaline Mx1 in HEK-293T cells. Forty-eight hours after transfection of the expression plasmid, cell extracts were subjected to immunoblotting, and cell monolayers were fixed for immunocytochemistry. Data are representative of two separate experiments. Blots were probed for both bubaline Mx1 and  $\beta$ -actin, and all of them displayed a strong band at ~40 kDa, the expected MW of  $\beta$ -actin. ++: very positive result; +: positive result and -: negative result.



**Figure 3:** Immunoblotting profiling of 12 mAbs against total protein extracts from transfected RFP-positive (V5-bbMx1-expressing) HEK-293T cells. See text for key.

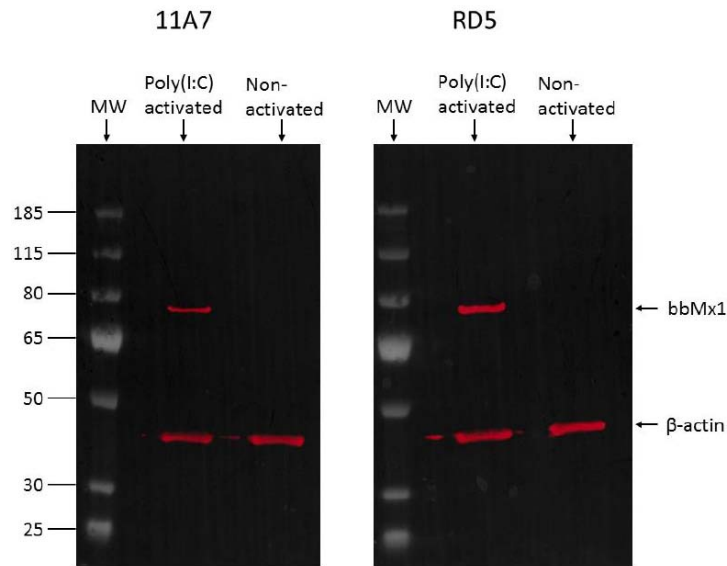
strongest signals (11A7 and RD5) and proteins extracted from poly(I:C) activated versus inactivated *Bubalus bubalis* primary cells. Both mAb generated strong signals of expected size only with activated cells. As expected, no signal was seen in non-activated bubaline cells (Figure 4).

Immunocytochemistry studies revealed comparable results (Figure 5), with the absence of staining by mAb

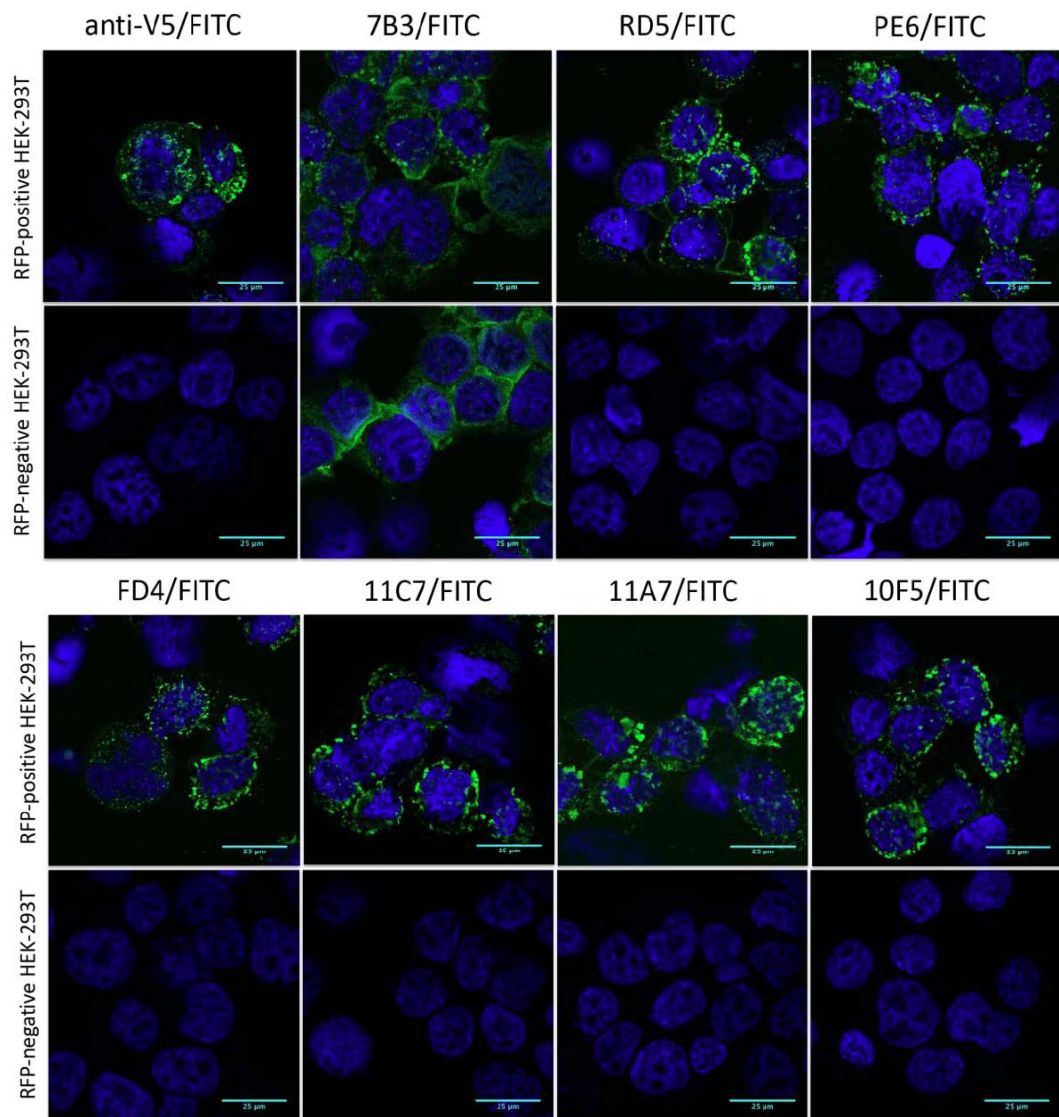
OB8 and nonspecific staining by 7B3. Besides, the other 8 mAbs displayed a strong signal, stronger than that retrieved from cells stained with the commercial anti-V5 epitope mAb.

#### Between-Mx1 Specificity

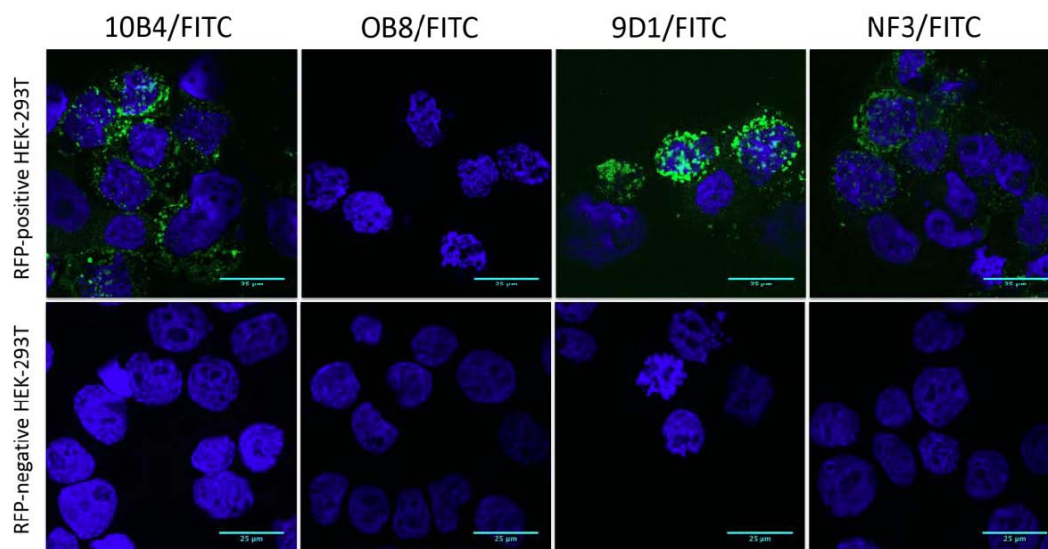
Immunoblots were then repeated using porcine- and equine V5-Mx1-expressing cells in order to examine



**Figure 4:** Immunoblotting profiling of 2 mAbs against total protein extracts of poly(I:C) activated versus inactivated *Bubalus bubalis* primary cells. See text for key.



(Figure 5). Continued.



**Figure 5:** Immunocytochemical characterization of 12 mAbs using RFP-positive (V5-bbMx1-expressing) and RFP-negative (non transfected) HEK-293T cell monolayers. See text for key.

**Table 2: Specificity of Anti-Buffalo-Mx1 Monoclonal Antibodies in Immunoblots**

mAbs ↓	<i>Sus scrofa</i> V5-Mx1			<i>Equus caballus</i> V5-Mx1		
	RFP-positive cells		Control cells	RFP-positive cells		Control cells
	~75 kDa	~50 kDa		~75 kDa	~50 kDa	
anti-V5 tag	+	-	-	+	-	-
FD4	-	-	-	-	-	-
PE6	-	-	-	-	-	-
RD5	-	-	-	-	-	-
7B3	++	+	+	++	+	+
10B4	+	-	-	+	-	-
11A7	+	-	-	-	-	-
10F5	-	-	-	-	-	-
9D1	++	-	-	-	-	-
11C7	+	-	-	-	-	-

Plasmids used for transfection were either pDA657RA/V5-ssMx1 (*Sus scrofa*) or pDA657RA/V5-ecMx1 (*Equus caballus*). ++: very positive result; +: positive result; -: negative result.

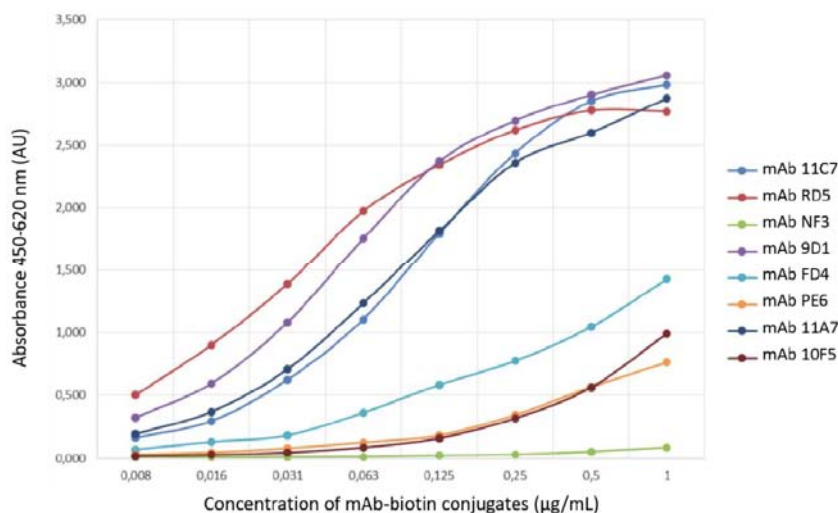
the possible cross-reactivity of the 8 anti-buffalo-Mx1 antibodies by immunoblotting (Table 3). Three functional categories were revealed: (i) FD4, PE6, RD5 and 10F5 mAbs did not cross-react, (ii) 7B3 and 10B4 cross-reacted with both non-bubaline V5-Mx1 and (iii) 11A7, 9D1 and 11C7 cross-reacted with pig V5-Mx1 only. Again, mAb 7B3 also detected a ~50 kDa protein in both transfected and control cells.

### Feasibility Study of a Sandwich-ELISA

In order to carry out a first feasibility study of the ELISA tool targeted, we compared the potential of all

possible pairs of ligands in a prototype sandwich format. On the basis of characterisation studies, three anti-buffalo-Mx1 mAbs were not enrolled in the experimental design, either because V5-recombinant bbMx1 produced by mammalian cells was only weakly detected (OB8) or because of a lack of specificity (10B4 and 7B3). Conversely, mAb NF3 initially developed for targeting bovine Mx1 was joined to the set of mAbs being tested. First, the 8 mAbs were biotinylated and used as detection ligands of coated His<sub>6x</sub>-bbMx1 in a direct ELISA format (Figure 6). Two affinity categories clearly appeared, with 11C7, RD5,





**Figure 6:** Typical titration of the 8 biotinylated candidate mAbs against recombinant His<sub>6x</sub>-bbMx1 by direct ELISA. The binding was detected by streptavidin-HRP conjugate, the latter being revealed using the chromogenic substrate tetramethylbenzidine. See text for key.

**Table 3: Comparison of the 64 Possible Capture/Detection Pairs in a Prototype Sandwich-ELISA aimed at Quantitating Water Buffalo Mx1**

Detection by ↓	Coating by 11C7		Coating by RD5		Coating by NF3		Coating by 9D1		Coating by FD4		Coating by PE6		Coating by 11A7		Coating by 10F5	
	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL	10 ng/mL	0 ng/mL
11C7-biotin 0,125 µg/mL	0,131	0,081	0,475	0,037	0,255	0,036	0,434	0,040	0,570	0,050	0,503	0,042	0,243	0,038	0,098	0,042
RD5-biotin 0,125 µg/mL	0,489	0,033	0,078	0,024	0,554	0,022	0,652	0,023	0,158	0,024	0,173	0,024	0,381	0,028	0,123	0,025
NF3-biotin 1 µg/mL	0,112	0,104	0,064	0,054	0,189	0,187	0,080	0,064	0,086	0,065	0,081	0,060	0,104	0,071	0,113	0,087
9D1-biotin 0,125 µg/mL	0,209	0,029	0,346	0,022	0,248	0,021	0,107	0,023	0,407	0,023	0,390	0,023	0,096	0,023	0,068	0,025
FD4-biotin 1 µg/mL	0,246	0,046	0,074	0,039	0,328	0,031	0,402	0,049	0,138	0,050	0,144	0,048	0,253	0,054	0,102	0,051
PE6-biotin 1 µg/mL	0,142	0,053	0,054	0,042	0,148	0,022	0,213	0,050	0,088	0,052	0,092	0,057	0,158	0,047	0,076	0,046
11A7-biotin 0,125 µg/mL	0,140	0,029	0,232	0,025	0,170	0,019	0,078	0,025	0,268	0,025	0,263	0,029	0,078	0,025	0,058	0,025
10F5-biotin 1 µg/mL	0,244	0,108	0,279	0,068	0,255	0,097	0,286	0,096	0,309	0,094	0,336	0,102	0,213	0,084	0,164	0,124

Reported numbers are mean absorbances from triplicate assays of recombinant buffalo Mx1 solutions (either 10 or 0 ng/mL). Stained boxes correspond to categories of absorbances at 10 ng/mL with either >0,5 (black), between 0,3 and 0,5 (dark grey) and between 0,1 and 0,3 (light grey). The dotted boxes indicate the capture/detection pairs generating an abnormally high background noise.

9D1 and 11A7 performing far better as detection ligands. Afterwards, the 64 possible capture/detection pairs were compared in a prototype sandwich-ELISA format (Table 3). In this experiment, the lower affinities displayed by mAbs NF3, FD4, PE6 and 10F5 as detection ligands (Figure 5) were compensated by increasing their concentration (1 vs. 0,125 µg/mL). With the development of a future diagnostic tool in mind,

four pairs were immediately disqualified because they generate too much background noise: 11C7/NF3, 11C7/10F5, PE6/10F5 and 10F5/10F5 (capture/detection). Besides, three functional categories were easily defined on the basis of the amplitude of the signal generated when a solution titrating 10 ng/ml His<sub>6x</sub>-bbMx1, i.e. corresponding to the basal level of MxA in healthy adults [23] was tested (Table 3). Fifteen

of the capture/detection pairs generated a signal at least six times higher than the background noise generated by PBS when a solution titrating 10 ng/ml of bbMx1 was introduced. Among them, the readout obtained from the pairs NF3/RD5, 9D1/RD5, FD4/11C7 and PE6/11C7 was at least ten times that of the background.

## DISCUSSION

Globalization has resulted in enhanced trade in livestock and livestock products all over the world [24]. In particular, water buffaloes are increasingly moved large distances across the Greater Mekong Sub-region in order to satisfy the growing demand for beef in China [25]. This extensive movement of livestock implies a risk of spreading infectious diseases within the region. This threat would be best controlled by addressing disease at its source, i.e. by preventing diseased animals from moving. In this context, the development of a test allowing the early detection of viral infection appears of primordial importance.

Mx1 concentration is regulated by type I/III IFNs in peripheral blood cells. Moreover, viruses can directly activate transcription of Mx1 mRNA [26]. Therefore a rapid determination of Mx1 expression could be considered a powerful strategy for the diagnostic of viral diseases in general. Besides, Mx proteins could be used as biomarkers for differentiating viral from bacterial infections hence guiding optimal use of antibiotics in medicine [27]. Choosing an appropriate antigen is an important part of hybridoma development [28]. As the native buffalo Mx1 proteins were difficult to obtain, recombinant Mx1 was used as antigen to generate the intended mAbs. In the current study, the whole recombinant protein bbMx1 was expressed successfully in *E. coli* with a His-tag at the N-terminus. A purification procedure employing immobilized metal-affinity chromatography allowed rapid production of large quantities of pure recombinant Mx1. As eukaryotic recombinant proteins derived from *E. coli* do not display their native tridimensional form, it was vital to develop appropriate characterization methods to avoid overvaluation of candidate mAbs dedicated to the detection of the said Mx1 in clinical samples. Control cells and cells transfected with different vectors were produced and enrolled in the screening, and the results proved that the procedure was effective.

In this study, mAbs 11C7, RD5, NF3, 9D1, FD4, PE6, 11A7 and 10F5 were specific to bbMx1, as determined by western blot, immunocytostaining

analysis and direct ELISA. Moreover, combining NF3 with RD5, 9D1 with RD5, FD4 with 11C7 or PE6 with 11C7 generated a strong signal in a prototype sandwich-ELISA. The results suggest that the mAbs developed and characterized here provide an excellent starting point for developing diagnostic tools for detecting viral infections in the water buffalo, whatever using immunoblotting, immunocytostaining or sandwich-ELISA.

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## SUPPLEMENTAL MATERIAL

The supplemental material can be downloaded from the journal website along with the article.

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