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Production of monoclonal antibodies binding to the VP7 protein of African horse sickness virus

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ABSTRACT

Monoclonal antibodies (MAbs) against AHSV were produced by immunising BALB/c mice with AHSV serotype 9 and six clones able to recognize specifically the VP7-AHSV with a strong reactivity were selected. The specificity of the MAbs was assessed in i-ELISA against a commercial VP7-AHSV and in immunoblot against a home-made VP7-AHSV, expressed by a Baculovirus expression system; potential cross-reactions with related orbiviruses (Bluetongue virus and Epizootic Haemorrhagic Disease virus) were investigated as well. One of the six MAbs selected, MAb 7F11E14, was tested in direct immunofluorescence and reacted with all nine AHSV serotypes, but didn't cross-react with BTV and EHDV. MAb 7F11E14 was also used to develop a competitive ELISA and was able to detect AHSV antibodies in the sera of AHS infected animals.

1. Introduction

African horse sickness (AHS) is a non-contagious, infectious disease of equids transmitted by haematophagous vectors belonging to the *Culicoides* genus (Diptera: Ceratopogonidae) (World Organisation of Animal Health, 2017; Carpenter et al., 2010; Mellor and Hamblin, 2004). Although zebra and donkeys rarely exhibit clinical signs, the effects of the disease, particularly in susceptible populations of horses, can be devastating and mortality rates for this species may exceed 90%. The AHS virus (AHSV), the causative agent of the disease, is a double stranded RNA virus which belongs to the family *Reoviridae*, genus *Orbivirus* (MacLachlan and Guthrie, 2010) and shares many morphological and structural characteristics with the other members of the genus such as Bluetongue virus (BTV) and Epizootic Haemorrhagic Disease virus (EHDV) (World Organisation of Animal Health, 2017; Mellor and Hamblin, 2004). So far, nine AHSV serotypes have been serologically identified, occurring mainly in eastern and southern Africa where the disease is endemic. Outbreaks occurred outside these regions on several occasions as in North Africa (1965, 1989–1990), in Middle East (1959–1961) and in southern Europe (Spain, 1966, 1987–1990 and Portugal 1989). Because of the high correlation with BTV and its presence in countries bordering Europe, AHSV might really represent the emergence that Europe has to deal with in the next future. The chance that the virus could enter Europe and become endemic is not so remote. For these reasons the International Institutions have issued recommendations and protocols in order to have a functional early warning system throughout Europe based also on the availability of rapid and reliable diagnostic methods in the laboratories.

The World Organization for the Animal Health (OIE) recommends ELISA, complement fixation and virus neutralization as prescribed tests for the serological screening of the susceptible population (World Organisation of Animal Health, 2017). Among these assays, ELISA proved to be a good method for the detection of anti-AHSV group-reactive antibodies, targeting the Viral Protein 7 (VP7).

The availability of AHSV-specific monoclonal antibodies (MAbs) paves the way to develop or improve diagnostic methods. This study describes the production and characterization of MAbs specific for AHSV-protein VP7 useful in immunofluorescence and ELISA for the diagnosis of AHSV.

2. Material and methods

2.1. AHSV whole antigen production

Vero cells were infected with $10^{4.5}$ $TCID_{50}$/ml of African Horse Sickness virus serotype 9 (AHSV-9) diluted in Minimum Essential Medium (MEM) (Biowest, SAS Nauillé) in serum free condition and incubated at +37 °C. The viral suspension was harvested at the 90% of

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cytopatic effect and centrifuged at 13,500 *g* for 30 min at +4 °C; the pellet was diluted 1:5 in sterile 0.01 M phosphate buffered saline, pH 7.2 (PBS), and stored at −70 °C until use. After thawing, the viral suspension was purified according to Lelli et al. (2003). The same protocol was adopted to prepare negative control antigen using not infected Vero cells.

2.2. Recombinant VP7-AHSV1 production

The entire sequence of segment 7 serotype 1 (GenBank Genome Accession number AM883171.1), encoding the major capsid protein (VP7) of African horse sickness virus, was cloned into the transfer vector pENTR1A (GenScript); pENTR1A-VP7-AHSV1 construct was made by amino-terminal fusion of a six-histidine residue tag and a V5 tag and was expressed in baculovirus system following the manufacturer's specifications (BaculoDirect™ N-Term Linear DNA, Invitrogen) and using the Gateway® technology. *Spodoptera frugiperda* cell clone 9 (Sf9) was used to propagate recombinant baculoviruses (AcMNPV-VP7-AHS1). Sf9 cells were infected at a multiplicity of infection (MOI) of 1 PFU/cell and incubated at 27 °C for 3 days. Then, infected Sf9 cells were harvested, lysed and centrifuged at 3250 *g* for 10 min at 4 °C. The pellet was purified on sucrose gradient as described by Maree and Paweska (2005), and solubilized with Sarcosyl® according to Rutkowska et al. (2011).

Rec-VP7-AHSV1 protein expression was evaluated by SDS-PAGE and western blotting using the anti-V5 HRP antibody (Life Technologies) and one of the produced MAbs specific for VP7-AHSV.

2.3. Immunization of mice and production of monoclonal antibodies (MAbs)

The animal experimentation was carried out in compliance with the Italian national law in force until 2014, i.e. Legislative Decree 27 January 1992, N. 116 (Anonymous, 1992) implementing Directive 86/609/EEC of the Council of the European Communities on the protection of animals used for experimental and other scientific purposes (European Commission, 1986). The protocol was approved by the Italian Ministry of Health with number 5146 of 26/04/2012.

Four female BALB/c mice were inoculated intraperitoneally on day 0 and 14 with 50 µg/mouse of AHSV-9 whole antigen mixed in a 1:1 ratio with complete Freund's adjuvant and Freund's incomplete adjuvant, respectively. On days 28 and 42, mice were immunized with 50 µg of viral antigen diluted in sterile PBS. Mice sera were tested by indirect ELISA (i-ELISA) (Harlow and Lane, 1988) *vs* AHSV-9 whole antigen to check antibody titers; animals with the highest serum titers were euthanized.

Cell fusion and hybridoma cloning were performed according to Luciani et al. (2006). Briefly, B lymphocytes were fused with Sp2/O-Ag-14 mouse myeloma cells (ATCC CRL-1581™), using 50% polyethylene glycol solution (PEG 1550, Serva Feinbiochema GmbH & Co). Hybridomas were cultured in Dulbecco's Modified Eagle Medium (Sigma) containing 20% fetal bovine serum (Euroclone) and 50X HAT Media supplement (HybriMax®, Sigma); antibody-secreting hybridomas were cloned by limiting dilution method.

Hybridomas were screened by i-ELISA using AHSV-9 whole antigen (10 µg/ml) and the negative control (not infected Vero cells) (10 µg/ml); selected clones were amplified *in vitro* by serial cultures and their supernatants collected and isotyped using a commercial kit (MouseTyper® Isotyping Panel, Biorad).

2.4. MAbs characterization by ELISA

MAbs characterisation was performed in i-ELISA to investigate reactivity *vs* rec-VP7-AHSV1 and potential cross-reactions with related *Orbivirus* as Bluetongue virus (BTV) and Epizootic Haemorrhagic Disease virus (EHDV).

BTV and EHDV purified whole viruses and rec-VP7-AHSV1 (IZSAM), rec-VP7-BTV2 (IZSAM) and rec-VP7-EHDV1 (IZSAM) diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to a final concentration of 5 µg/ml, were coated into 96-well microplates (PolySorp, Nunc) and incubated overnight at +4 °C. After washing and blocking with 1% yeast extract in PBS containing 0.05% Tween 20 (PBS-T) at room temperature (RT) for 1 h, MAbs supernatants were added and incubated for 1 h at RT. The plates were then washed with PBS-T and incubated with ECL™ Anti-mouse IgG-HRP from sheep (GE Healthcare) at RT for 30 min. After further washes, 100 µl/well of TMB chromogen substrate (Sigma) were added into each well for 30 min, the reaction stopped with 0.5 N sulphuric acid and the optical density values were measured at 450 nm (OD_{450}).

MAbs reactivity *vs* rec-VP7-EHDV and rec-VP7-AHSV was also assessed using, respectively, two commercial ELISA kit (LSIVET EHDV Blocking, LSI, and Ingezim AHSV Compac Plus, Ingenasa) and the procedure described above.

Different levels of antigen-antibody reaction *vs* the rec-VP7-AHSV of the commercial kit were expressed based on values of OD_{450} as following described: strong reactivity ($OD_{450} \geq 2.000$), mild reactivity ($2.000 \geq OD_{450} \geq 1.000$), weak reactivity ($1.000 \geq OD_{450} \geq 0.300$), no reaction ($OD_{450} \leq 0.300$). In order to develop a c-ELISA, the competition of MAbs and serum immunoglobulins for immunogenic epitopes was assessed using an AHSV9 horse positive serum (SN titre 1:320) at different dilutions (1:10, 1:40, 1:80) (50 µl/well), a negative horse serum diluted 1:10 (50 µl/well) and the ECL™ Anti-mouse IgG-HRP (50 µl/well).

2.5. MAbs characterization by western blotting

MAbs that showed strong and mild reactivity ($OD_{450} \geq 1.000$) in i-ELISA *vs* the commercial rec-VP7-AHSV and were suitable for the c-ELISA were characterized by western blotting techniques. AHSV whole antigen, rec-VP7-AHSV1 (IZSAM), rec-VP7-BTV2 (IZSAM) and rec-VP7-EHDV1 (IZSAM), at the concentration of 5 µg per well, were separated by SDS-PAGE. using NuPage® 12% Bis-Tris pre-cast gels (Life Technologies). Immunoblotting was performed according to Di Febo et al. (2018). The molecular weight of the protein bands was determined by comparison with the Novex® Sharp Protein Standard (Life Technologies). Analyses were performed using Quantity One® Software Version 4.3 (Biorad).

2.6. MAbs purification and HRP-conjugation

Hybridomas producing MAbs reacting both with native and denatured VP7-AHSV were grown in DMEM containing 10% FBS (v/v) (Sigma), 2 mM glutamine (Sigma), 100x penicillin-streptomycin –amphotericin (Sigma), 50 mg/mL gentamicin (Sigma), and 10,000 IU/mL nystatin (Sigma). Supernatants were purified by affinity chromatography using a protein G column (HiTrap™ Protein G HP, 5 ml, GE Healthcare) (IgG1 isotype) or a protein A column (HiTrap™ rProtein A FF, 5 ml, GE Healthcare) (IgG2a and IgG2b isotypes), according to manufacturer's instructions. After elution, buffer solution was replaced with PBS using molecular filters with a cut-off value of 100 kDa (Amicon Ultra-15, Millipore) and MAb concentration was determined spectrophotometrically (280 nm). Purified MAbs were conjugated with horseradish peroxidase (Sigma) as described by Nakane and Kawaoi (1974) for the use in competitive ELISA.

2.7. Sera

Three hundred-and six horse sera, negative for AHSV, were collected during the national surveillance plan for Equine Infectious Anaemia in Italy; 25 sera were collected from AHSV naturally infected horses in Namibia (SN titres ranging from 1:10 to 1:320). Sera from 9 guinea pigs experimentally infected with the 9 AHSV serotypes (SN

titres ranging from 1:20 to 1:640), 8 guinea pigs experimentally infected with the 8 EHDV serotypes (SN titres ranging from 1:40 to 1:1280), 5 sera from healthy guinea pigs, 4 sera from sheep and bovine naturally infected with BTV serotypes 2, 4, 6 and 8 were also used. Animal experimentation was carried out in compliance with Italian national law (Legislative Decree 26/2014) implementing Directive 2010/63/EU of the Council of the European Communities on the protection of animals used for scientific purposes (Anonymous, 2014, 2010). Ethical approval was obtained from the Italian Ministry of Health (protocol number 113 of the 19/12/2014-PR). All the positive sera were tested with the commercial ELISA kit Ingezim AHSV Compac Plus (Ingenasa).

2.8. Competitive ELISA

The rec-VP7-AHSV1 (IZSAM), diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to a final concentration of 8 µg/ml, was dispensed (50 µl/well) into 96-well microplates (Polysorp, Nunc) and incubated overnight at +4 °C in a humidity chamber. The plates were washed once with PBS-T and blocked with 3% skimmed milk at +37 °C for 30 min. After three washes with PBS-T, 50 µl/well of blocking solution (MAb Control) and 50 µl/well of each serum diluted 1:5 in blocking buffer were added for 1 h at +37 °C. Plates were then washed and incubated at RT for 1 h with 50 µl/well of each MAb-HRP diluted in PBS-T. After further washes, 100 µl of TMB were dispensed into each well, and the plates were incubated at RT for 30 min. The reaction was stopped by adding 50 µl/well of 0.5 N sulphuric acid. Optical densities (OD_{450}) were measured and converted to B/B_0% values using the following formula: (OD_{450} sample serum / OD_{450} MAb Control) x 100.

The cut-off of the c-ELISA was calculated using receiver operator characteristic (ROC).

The analytical sensitivity of the c-ELISA was assessed by diluting an AHSV-9 positive horse serum (SN titre 1:320) from 1:10 to 1:1280. The analytical specificity was assessed by testing 8 guinea pig sera positive for the 8 EHDV serotypes and 4 sheep and bovine sera positive for BTV serotypes 2, 4, 6 and 8 (exclusivity) and 9 guinea pig sera positive for the 9 AHSV serotypes (inclusivity).

2.9. Immunofluorescence test

To confirm the reactivity of the MAb selected for the c-ELISA with all the AHSV serotypes and to evaluate its suitability for the use in a immunofluorescence test for the direct identification of the virus, cultures of Vero cells were infected with AHSV nine serotypes and mounted onto glass slides. Uninfected cells and BTV serotype 2 and EHDV serotype 2 infected cells were used as negative controls. Twenty-five µl of MAb diluted 1:50 in PBS were applied on the antigen-coated slides. After washes, antigen-MAb reaction was revealed using 25 µl of fluorescein-conjugated rabbit IgG fraction to mouse IgG (MP Biomedicals), diluted 1:80 in PBS containing 1% Evans Blue (Sigma-Aldrich). Slides were evaluated by fluorescence microscopy (20× magnification, Axio Lab. A1, Zeiss).

3. Results

3.1. Recombinant VP7-AHSV1 expression

The entire sequence of segment 7 serotype 1 encoding the major capsid protein (VP7) of AHSV was cloned into the vector pENTR1A. The pENTR1A-VP7-AHSV1 gene constructs was used to generate recombinant viruses in Sf9 cells and produce a VP7 recombinant fused to the six-histidine residue and V5 tag. To evaluate VP7 protein production, infected cells with AcMNPV-VP7-AHSV1 were harvested and whole cell lysates analysed by SDS-PAGE and Comassie staining and detected in immunoblotting with anti-V5-HRP specific antibody and MAb-HRP 7F11E14. A protein band with an approximate molecular mass of 40–42 KDa was observed (Fig. 1). The greater molecular weight (45 kDa) of the rec-VP7-AHSV1 in comparison with the molecular weight of the native VP7 (37 kDa) is due to the presence of the His-tag and V5-tag.

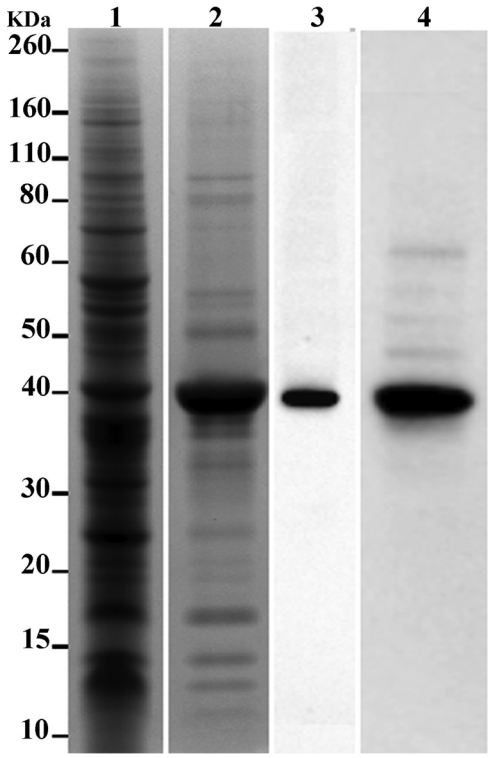

Fig. 1. Comassie stain of Sf9 raw lysate (lane 1) and purified rec-VP7-AHSV1 (lane 2). Western blotting of purified rec-VP7-AHSV1 with anti-V5-HRP antibody (lane 3) and MAb 7F11E14-HRP (lane 4). Rec-VP7-AHSV1 showed a molecular weight of 40–42 kDa.

3.2. Production and characterisation of MAbs

From cell fusion 59 hybridoma cell lines, secreting antibodies *vs* AHSV whole virus and showing no reaction against the negative control, were selected by i-ELISA. Among these, 19 MAbs not cross-reactive to BTV and EHDV (whole virus and recombinant VP7) showed strong and mild reactivity ($OD_{450} \geq 1.000$) when tested by i-ELISA using microplates coated with a recombinant VP7-AHSV of a commercial ELISA kit (Table 1).These 19 MAbs were also suitable for the use in c-ELISA and six of them (2D2E12, 2D10G10, 6E5B7, 7F11E11, 7F11E13, 7F11E14) showed the best results in terms of competition. Two of the 6 MAbs were IgG_1 anti κ (2D2E12 and 2D10G10), one was IgG_{2b} anti κ (6E5B7) and three IgG_{2a} anti κ (7F11E11, 7F11E13, 7F11E14).

Western blotting analysis showed that MAbs 2D10G10, 7F11E11, 7F11E13 and 7F11E14 recognized both the VP7 recombinant protein (band of 45 kDa) and the whole virus VP7 (band of 37 kDa), and didn't recognize rec-VP7-BTV2 (IZSAM) and rec-VP7-EHDV1 (IZSAM) (Fig. 2 and 3). The remaining two MAbs (2D2E12, 6E5B7) did not react with the denatured antigens (image not shown). Since the target epitopes of each MAb have not been evaluated, it could be possible that some of the selected MAbs could recognize the same or close epitopes. Results of the characterization of the 19 MAbs specific for VP7-AHSV are summarized in Table I.

3.3. Competitive ELISA

Among the selected MAbs anti-rec-VP7-AHSV, the most suitable for use in c-ELISA was MAb 7F11E14 (1:10,000 dilution). The cut-off

Table 1
Results of the characterization of MAbs selected for the c-ELISA. In bold MAbs with $OD_{450\ nm} \geq 2.000$. These MAbs did not cross-react with BTV (VP7 and whole virus), nor with EHDV (VP7 and whole virus).

	MAb	i-ELISA with a commercial VP7-AHSV ($OD_{450\ nm}$)	Isotype	Western blotting reaction
1	2D2E11	1.064	IgG_1 anti k	Yes
2	**2D2E12**	**2.686**	**IgG_1 anti k**	No
3	2D2E13	1.158	IgG_1 anti k	Yes
4	**2D10G10**	**2.436**	**IgG_1 anti k**	Yes
5	2D10G12	1.523	IgG_1 anti k	No
6	2E6F11	1.314	IgG_1 anti k	No
7	2E6F12	1.258	IgG_1 anti k	No
8	2E6F13	1.198	IgG_1 anti k	No
9	2E6F14	1.239	IgG_1 anti k	No
10	2E6G11	1.365	IgG_1 anti k	No
11	2E6G12	1.456	IgG_1 anti k	No
12	2F8D2	1.215	IgG_1 anti k	Yes
13	**6E5B7**	**2.391**	**IgG_{2b} anti k**	No
14	6F9D12	1.678	IgG_1 anti k	No
15	6F9D13	1.426	IgG_1 anti k	No
16	6F9D15	1.114	IgG_1 anti k	No
17	**7F11E11**	**2.053**	**IgG_{2a} anti k**	Yes
18	**7F11E13**	**2.027**	**IgG_{2a} anti k**	Yes
19	**7F11E14**	**2.052**	**IgG_{2a} anti k**	Yes

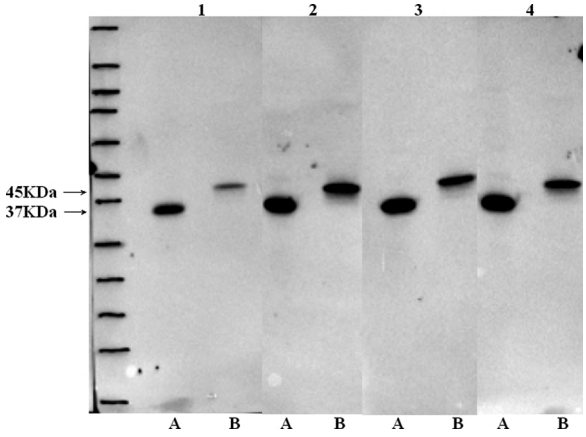

Fig. 2. Western blotting of MAbs 2D10G10 (1), 7F11E11 (2), 7F11E13 (3) and 7F11E14 (4) *versus* the AHSV whole virus (lane A) and rec-VP7-AHSV1 (IZSAM) (lane B). The native VP7 showed a molecular weight of 37 kDa; the rec-VP7-AHSV1 showed a greater molecular weight (45 kDa) due to the presence of the His-tag and V5-tag.

value, obtained using the ROC analysis, was 80.0% (B/B_0%). Serum samples with a B/B_0% value above or equal to 80.0% were considered negative, while those with a ratio below 80.0% were considered positive. All the AHSV positive sera tested positive in c-ELISA and all the negative sera tested negative (Fig. 5). In the evaluation of the analytical sensitivity, all the dilutions of the positive horse serum tested as positive in c-ELISA: the B/B_0% values ranged from 26.4% for the 1:10 dilution to 78.9% for the 1:1280 dilution. In the evaluation of the analytical specificity (exclusivity and inclusivity), sera positive for BTV and EHDV resulted negative in c-ELISA; on the contrary, all the guinea pig sera positive for the 9 AHSV serotypes tested positive.

3.4. Immunofluorescence test

To confirm specificity and reactivity of MAb 7F11E14, a direct antibody inmmunofluorescence test (IFA) was done. MAb 7F11E14 was used at a dilution giving optimal intensity of fluorescence with a minimum of background staining. Glass slides coated with Vero cells infected with the nine AHSV serotypes showed a granular fluorescence in the cytoplasm as small, “dust-like” particles. No fluorescence was observed in the slides coated with non-infected cells and cells infected with BTV serotype 2 and EHDV serotype 2 (Fig. 4).

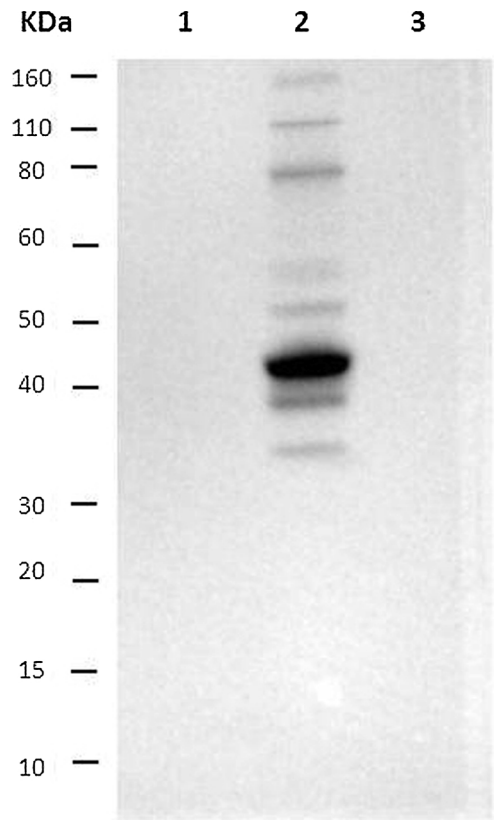

Fig. 3. Western blotting of the MAb 7F11E14 used to develop the c-ELISA *versus* rec-VP7-BTV2 (IZSAM) (lane 1), rec-VP7-AHSV1 (lane 2) and rec-VP7-EHDV1 (lane 3).

4. Discussion and conclusions

In recent years, certain diseases spread with increasing frequency outside the endemic areas of the African continent. The paradigmatic example is represented by the Bluetongue, which has demonstrated extraordinary ability to adapt, after its introduction, in the American continent (MacLachlan et al., 2013) and in the European continent, spreading from the Mediterranean area to United Kingdom and Scandinavia (Zientara and Sanchez-Vizcaino, 2013; Tollersrud, 2009; Saegerman et al., 2008). Other examples are AHS outbreaks in Spain (serotype 9, 1966; serotype 4, 1987–1990), and in Portugal (serotype 4, 1989) (World Organisation of Animal Health, 2017). These raids are capable of causing significant economic damages in terms of mortality of infected animals and livestock movement restrictions. To cope with such eventualities it is essential to have early warning systems based on efficient diagnostic techniques able to detect pathogens quickly and provide accurate results. For the development of such methods of diagnosis, standardized reagents are necessary. For this purpose, a panel of monoclonal antibodies against the AHS virus was produced.

In this study, 19 MAbs specific for the Viral Protein 7, the serogroup common antigen of the AHSV, and not cross-reacting *vs* related Orbiviruses, as BTV and EHDV, were selected and further characterized by SDS-PAGE and western blotting. Six MAbs (2D2E12, 2D10G10, 6E5B7, 7F11E11, 7F11E13, 7F11E14) showed the strongest reactivity

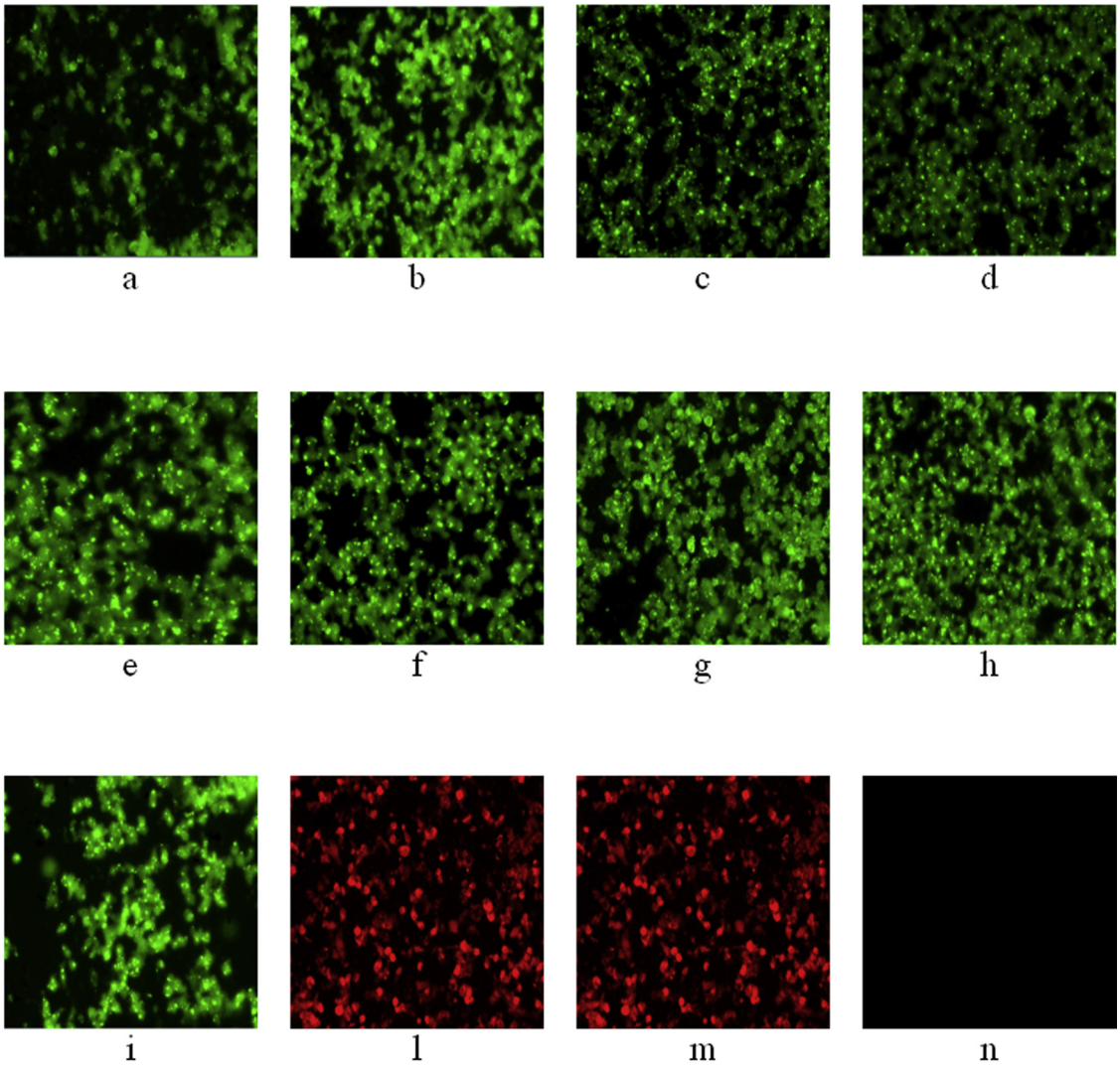

Fig. 4. Direct immunofluorescence with MAb 7F11E14. (A) AHSV serotype 1; (B) AHSV serotype 2; (C) AHSV serotype 3; (D) AHSV serotype 4; (E) AHSV serotype 5 (F) AHSV serotype 6; (G) AHSV serotype 7; (H) AHSV serotype 8; (I) AHSV serotype 9; (L) Bluetongue virus; (M) Epizootic Haemorrhagic Disease virus; (N) non infected Vero cells. The red fluorescence in the images "l" and "m" was probably due to aspecific reactions of the secondary antibody FITC-labeled with BTV and EHDV infected cells and to the Evans Blue counterstain. No corrections were applied to the images to eliminate the red fluorescence (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

with rec-VP7-AHSV (i-ELISA $OD_{450nm} > 2.0$). Blotting analysis showed that 4 of them recognized both the rec-VP7-AHSV and the native VP7 and the remaining 2 MAbs didn't recognize any band, probably because they recognize conformational epitopes of the VP7 (Russell et al., 2018; Kweon et al., 2003; Chuma et al., 1992). The 4 MAbs (2D10G10, 7F11E11, 7F11E13, 7F11E14), that reacted with both native and denatured form of the VP7-AHSV, can be used in the development of diagnostic tests using both the whole virus and the recombinant VP7-AHSV protein as antigens and for the direct identification of AHS virus in blood and tissues. One of selected MAbs, MAb 7F11E14, used to develop a c-ELISA, was able to discriminate among AHSV positive and negative sera. However the performances of the c-ELISA should be assessed with a greater number of AHSV positive and negative sera, according to OIE Manual (World Organisation of Animal Health, 2013). The MAb 7F11E14 was also tested in direct immunofluorescence, showing a high specificity for all nine AHSV serotypes and no cross-reactions with the most correlated Orbiviruses (BTV and EHDV) and with uninfected Vero cells. The immunofluorescence test is easy to perform and of rapid execution; it is often used as a screening test in the first phase of viral isolation, but sometimes it is the only method performed in the laboratories of emerging countries, where the disease is endemic and where tests that require more sophisticated instruments are unavailable. Finally, future studies will explore the use of MAbs anti-AHSV produced in this work in immunofluorescence, immunohistochemistry and sandwich ELISA for the direct identification of AHS virus in blood and tissues, and to better understand disease pathogenic mechanisms. Moreover VP7 protein shows a higher degree of conservation and is a very valuable candidate as a component for an AHSV cross-protective vaccine or to develop a sensitive and specific Luminex assay to detect anti-VP7 antibodies in horse serum (Sánchez-Matamoros et al., 2016).

Conflicts of interest

The authors have no competing financial interests or conflict of interests to declare.

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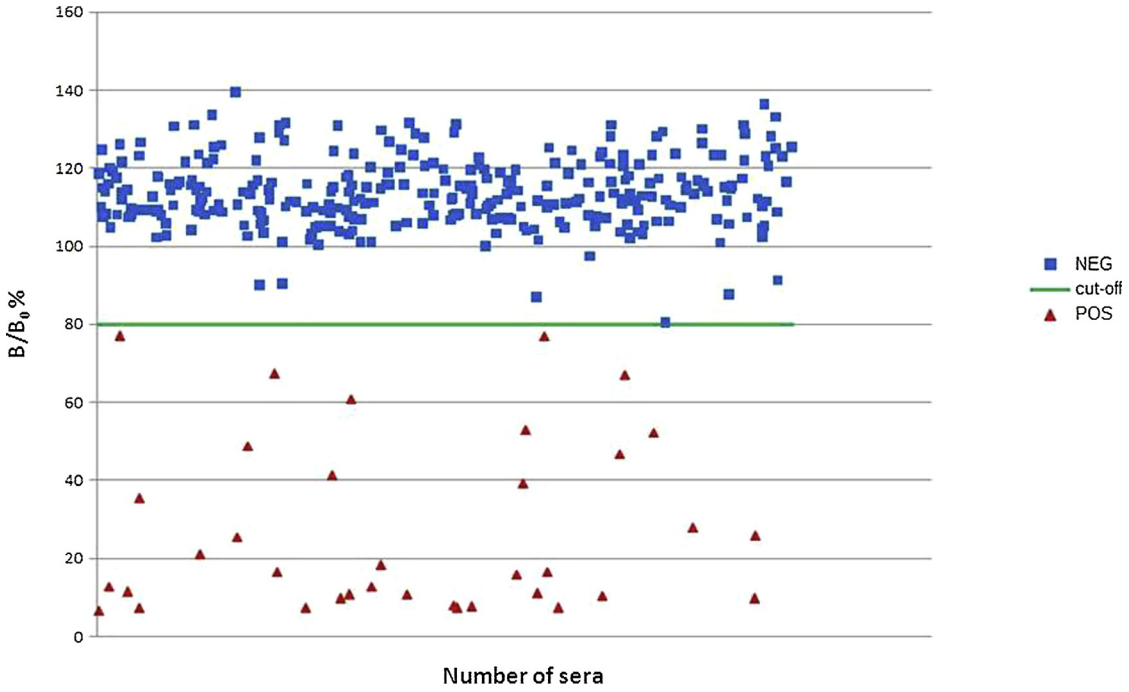

Fig. 5. Competitive ELISA: B/B0% values of AHSV negative (blue squares) and positive sera (red triangles). The green line represents the calculated cut-off value (80.0%) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

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References

Anonymous, 1992. Decreto legislativo 27 Gennaio 1992, n.116. Attuazione della direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. Official J. 40, 1–32.

Anonymous, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official J. Eur. Union L276/33.

Anonymous, 2014. Legislative Decree 4 March 2014 n. 26. Transposition of directive 2010/63/UE on the protection of animals used for scientific purposes. Official J. Ital. Republic n. 61 of 14/03/2014.

Carpenter, S., Philip, S., Mellor, P.S., Fall, A.G., Garros, C., Venter, G.J., 2010. African horse sickness virus: history, transmission, and current status. Annu. Rev. Entomol. 62, 343–358.

Chuma, T., Le Blois, H., Sanchez-Vizcaino, J.M., Diaz-Laviada, M., Roy, P., 1992. Expression of the major core antigen VP7 of African horse sickness virus by a recombinant baculovirus and its use as a group-specific diagnostic reagent. J. Gen. Virol. 73, 925–931.

Di Febo, T., Schirone, M., Visciano, P., Portanti, O., Armillotta, G., Persiani, T., Di Giannatale, E., Tittarelli, M., Luciani, M., 2018. Development of a capture ELISA for rapid detection of *Salmonella enterica* in food samples. Food Anal. Methods. https://doi.org/10.1007/s12161-018-1363-2.

European Commission (EC), 1986. Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Official J. L358, 1–28.

Harlow, E., Lane, D., 1988. In: Harlow, E., Lane, D. (Eds.), Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, NY, pp. 92–612.

Kweon, C.H., Kwon, B.J., Ko, Y.J., Kenichi, S., 2003. Development of competitive ELISA for serodiagnosis on African horse sickness virus using baculovirus expressed VP7 and monoclonal antibody. J. Virol. Methods 113, 13–18.

Lelli, R., Portanti, O., Langella, V., Luciani, M., Di Emidio, B., Conte, A.M., 2003. Produzione di un kit ELISA competitiva per la diagnosi sierologica della Bluetongue (Production of a competitive ELISA kit for the serological diagnosis of Bluetongue disease). Vet. Ital. 47, 5–13.

Luciani, M., Armillotta, G., Magliulo, M., Portanti, O., Di Febo, T., Di Giannatale, E., Roda, A., Lelli, R., 2006. Production and characterization of monoclonal antibodies specific for *Escherichia coli* O157:H7. Vet. Ital. 42 (3), 173–182.

MacLachlan, N.J., Guthrie, A.J., 2010. Re-emergence of Bluetongue, African horse sickness and other Orbivirus diseases. Vet. Res. 41 (6), 35–46.

MacLachlan, N.J., Wilson, W.C., Crossley, B.M., Mayo, C.E., Jasperson, D.C., Breitmeyer, R.E., Whiteford, A.M., 2013. Novel serotype of bluetongue virus, Western North America. Emerg. Infect. Dis. 19, 665–666.

Maree, S., Paweska, J.T., 2005. Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. J. Virol. Methods 125 (1), 55–65.

Mellor, P.S., Hamblin, C., 2004. African horse sickness. Vet. Res. 35, 445–466.

Nakane, P.K., Kawaoi, A., 1974. Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem. 22, 1084–1091.

Russell, B.L., Parbhoo, N., Gildenhuys, S., 2018. Analysis of conserved, computationally predicted epitope regions for VP5 and VP7 across three Orbiviruses. Bioinform. Biol. Insights 12, 1–12.

Rutkowska, D.A., Meyer, Q.C., Maree, F., Vosloo, W., Fick, W., Huismans, H., 2011. The use of soluble African horse sickness viral protein 7 as an antigen delivery and presentation system. Virus Res. 156 (1-2), 35–48.

Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue epidemiology in the european union. Emerg. Infect. Dis. 14, 539–544.

Sánchez-Matamoros, A., Beck, C., Kukielka, D., Lecollinet, S., Blaise-Boisseau, S., Garnier, A., Rueda, P., Zientara, S., Sánchez-Vizcaíno, J.M., 2016. Development of a microsphere-based immunoassay for serological detection of African Horse Sickness Virus and comparison with other diagnostic techniques. Transbound. Emerg. Dis. 63 (6), 270–277.

Tollersrud, T., 2009. Bluetongue - Europe (06): Norway, First Cases Detected. Archive No. 20090221.0729. http://www.promedmail.org.

World Organisation of Animal Health (Office International des Épizooties: OIE), 2013. Chapter 1.1.6 principles and methods of validation of diagnostic assays for infectious diseases. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris, pp. 1–16.

World Organisation of Animal Health (Office International des Épizooties: OIE), 2017. Chapter 2.5.1 African horse sickness. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris, pp. 1–16.

Zientara, S., Sanchez-Vizcaino, J.M., 2013. Control of Bluetongue in Europe. Vet. Microbiol. 165, 33–37.