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Veterinary Microbiology 46 (1995) 327–334

veterinary microbiology

Comparison of native and subunit antigens as ELISA reagents for the detection of antibodies against scabby mouth virus

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Accepted 8 March 1995

Abstract

A simple ELISA test to detect antibodies against scabby mouth virus (SMV) has been developed. Native whole virions and subunits of SMV generated by boiling the virus in the presence of sodium dodecyl sulphate (SDS) detergent and β-mercaptoethanol were compared as ELISA assay reagents using naive and hyperimmune sera from sheep and rabbits. Approximately 2×10^4 intact virus particles per microtiter well were required to generate a positive to negative signal of 0.8:0.3 ELISA O.D. units when the serum was used at a dilution of 1/100. In contrast, total subunit antigen generated by disrupting and coupling of 250–500 virions per well provided a signal ratio of 1.58:0.3 ELISA O.D. units at a serum dilution of 1/250. Total subunit antigens were therefore 400 times more economical to use than intact virions. In addition, subunit antigens could be readily bound to microtiter plates without the need for removal of the SDS. Secondly, it was not necessary to block non-specific binding sites on the plate with blockers such as gelatin and skim-milk, thereby shortening the time needed to complete the ELISA assay. The total subunit antigen ELISA test was used to detect seroconversion in new born lambs where there was an occurrence of SMV infection in housed sheep. Three bleeds were taken at fortnightly intervals and the ELISA results showed that 9 out of 15 lambs were sero-positive for all bleed points. Four of the lambs showed a sequential rise in titer while only one lamb failed to seroconvert. The subunit ELISA test can therefore provide a very economical and sensitive test for the detection of antibody reactivity against SMV.

Keywords: Scabby mouth virus; Sheep; Detection; Antigen – native – subunit

1. Introduction

Scabby mouth virus is a member of the parapox family and the aetiological agent of contagious ecthyma (CE). This highly contagious and eruptive skin condition is usually

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localised around the mouth, nose, thigh and udder of sheep. Currently, a live SMV vaccine is used for the control of CE but this represents a major source of contaminating virus in the environment and probably a contributory factor in carrier rates of the virus in the sheep population. Early clinical signs of CE can be misdiagnosed as bluetongue, sheeppox or foot and mouth disease. The development of improved serological tests for the detection of antibodies against SMV would help in the identification of sheep exposed to SMV. Although several tests including gel precipitation (Trueblood et al., 1963) and complement fixation (Glover, 1933) have been used for the detection of anti-SMV antibodies, they consume relatively large quantities of antigen with comparatively low levels of sensitivity. More recently, an ELISA using detergent released antigen has been described by McKeever et al. (1987) for the assessment of humoral antibody response of sheep to orf (SMV) virus infection. They also describe the use of western blotting to identify subunit antigens which immunoreact with serum antibodies from experimentally infected sheep. We report in this paper an extension of these studies by comparing the utility of native virus and denatured viral subunits as ELISA reagents for the detection of antibodies against SMV.

2. Materials and methods

2.1. Growth and purification of virus

Primary lamb testes cells maintained in modified Eagles medium (MEM) containing 2% (v/v) bovine calf serum were infected with SMV at a multiplicity of infection (m.o.i.) of 0.05. Infected cells were harvested by scraping with a rubber policeman and the cell pellet recovered by low speed centrifugation (250 *g*). The cell pellet was sonicated to release virus particles and the debris removed by low speed centrifugation. Virus in the culture supernatant fluid was recovered by centrifugation at 5000 *g*. This pellet was further purified by buoyant density centrifugation on a continuous sucrose gradient (20–50% w/v) made up in TEN buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 5 mM EDTA). The virus was recovered as a turbid band around the 40–45% (w/v) gradient mark.

2.2. Hyperimmune antisera

Rabbits and sheep were vaccinated with heat-killed (60°C, 1 h) purified SMV (10^6 TCID per ml) emulsified in complete Freund's adjuvant. Two further booster doses emulsified in incomplete Freund's adjuvant were administered at fortnightly intervals. Blood was collected before vaccination and two weeks after the last booster dose had been given. Blood samples were allowed to clot and serum was removed and diluted 1 in 5 in TSGM (25 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5% (v/v) glycerol, 0.02% (w/v) merthiolate) before storage at −20°C.

2.3. Field antisera

An occurrence of SMV infection in shedded pregnant sheep permitted the collection of blood from new born lambs to assess their ELISA response against subunit antigen. Blood

was collected randomly from 15 lambs on 3 occasions at 2 week intervals. The first blood was taken when the lambs were between 3–4 weeks old.

2.4. ELISA

Intact virus (IV) and subunit ELISA (Enzyme linked immunosorbent assays) were carried out using sucrose gradient purified native SMV antigen and denatured subunit antigens respectively. For IV-ELISA, a suspension of native SMV (10^7 TCID /ml) was first diluted 1/50 in coating buffer [CB: 50 mM bicarbonate buffer (pH 9.6)] and 50 ul aliquots were added in triplicate to each well of a 96-well microtiter plate (Flow Laboratories). Total subunit antigens (TSA) were generated by boiling sucrose gradient purified SMV (10^7/ml) in Laemmli reducing mixture [60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) merthiolate] and appropriate aliquots of this mixture were then diluted 1/250 with CB and coated to microtiter plates. Antigens were allowed to bind to the solid phase at 37°C for 1 h or overnight at 4°C. The plates were washed in TSTw [20 mM Tris-HCl pH 7.4; 0.15 M NaCl and 0.01% (v/v) Tween 20] followed by the sequential addition of antisera, HRP conjugated anti-species specific antisera (KPL) and substrate [40 mM o-phenylene diamine in 50 mM disodium hydrogen phosphate/25 mM citric acid buffer; pH 5]. Titration for optimal antigen binding concentrations were conducted by further serial two-fold dilutions of either IV or TSA in CB.

2.5. SDS-PAGE and Immunoblotting

Protein electrophoresis was carried out in a mini Hoeffer gel system under reducing conditions as described by Laemmli (1970). All samples were heated at 100°C for 5 min in Laemmli reducing mixture before loading onto a 10% (w/v) acrylamide running gel with a 4% (w/v) stacking gel. Electrophoresis was carried out at a constant voltage of 120V until the bromophenol blue tracking dye had moved to the bottom of the gel. Proteins were electrophoretically transferred from the acrylamide gel to nitrocellulose in a BioRad Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA) under conditions essentially similar to those described by Chin and Turner (1990) except that isopropanol was used instead of methanol. Immunoblots were blocked by incubation with high salt-Tween [25 mM Tris-HCl, pH 8.9; 0.15 M NaCl and 0.5% (v/v) Tween 20], washed in TSTw and then reacted with antisera diluted in TSTw. Antibodies which bound to nitrocellulose were located by reaction with their respective horseradish peroxidase (HRP) conjugated anti-species specific antibody (KPL). Immunoblots were developed with freshly prepared DAB substrate [20 mM Tris-HCl pH 7.4; 0.015 M NaCl; 1.4 mM 3,3-diaminobenzidine; 0.03% (v/v) H_2O_2].

3. Results

3.1. Immunoreactivity of hyperimmune sera

The electrophoretic profile of Coomassie Blue-stained polypeptides of SMV is shown in Fig. 1 (lane B). There were more than 30 subunit bands with the most prominent ones

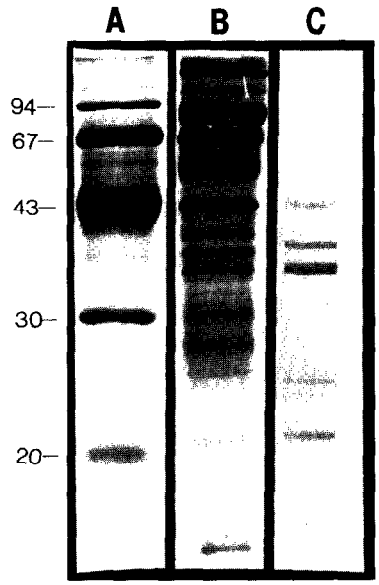

Fig. 1. Electrophoretic and immunoblot profiles of SMV. 15 μl of Laemmli treated virus was loaded in lane B and the polypeptide bands stained with Coomassie blue. An immunoblot of SMV subunits that had been electrophoretically transferred to nitrocellulose and reacted with hyperimmune sheep sera is depicted in lane C. Control serum from the same sheep collected prior to vaccination did not react in the immunoblots. Molecular weight standards are shown in lane A.

located at 110, 82–84, 67–69, 54, 43, 39.5, 38, 36, 34, 31, 28 and 15 kDa and less strongly stained bands at 60.5, 48.5, 42, 33, 25, 23, 17 and 19 kDa. Sera from hyperimmune sheep reacted in western blots against major bands located at 82–84, 43, 38, 36, 25 and 23 kDa (Fig. 1, lane C).

3.2. *Titration of native SMV antigen*

The initial dilution of 1/50 (No. 1 in Fig. 2) delivered an equivalent amount of 2×10^4 $TCID_{50}$ intact virus per microtiter well. Although this gave a reasonably good level of discrimination between pooled positive and pooled negative sheep sera (at a dilution of 1/100), the reactivity decreased rapidly with each subsequent doubling dilution. There was virtually no difference between positive and negative sera by the sixth doubling dilution (1/1600) when the assay was done with about 600 $TCID_{50}$ virus per well.

3.3. *Titration of total subunit SMV antigen*

Fig. 3 shows the ELISA results when total subunit SMV was used as the ELISA reagent. The first dilution of TSA was 1/250 (No. 1, Fig. 3) which effectively delivered the equivalent of 2000 $TCID_{50}$ virus per well. Dilutions of TSA at 1/1000 and 1/2000 were found to be optimal when sheep sera were being used at a dilution of 1/250. From then on, ELISA reactivity decreased linearly with each doubling dilution, reaching the limits of detection at No. 8 (15 $TCID_{50}$ subunit virus equivalent per well). TS antigen dilutions

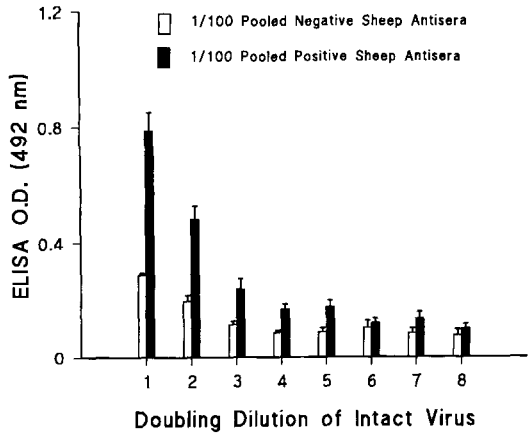

Fig. 2. Titration of intact SMV virions as an ELISA reagent. Doubling dilutions of intact virus were coupled in triplicate to microtiter plates as described in Materials and Methods. Pooled negative (from naive sheep prior to vaccination) and positive (from SMV-hyperimmune sheep) sera were added at a final dilution of 1/100.

between 1/500 (1000 $TCID_{50}$ subunit virus equivalent/well) to 1/2000 (250 $TCID_{50}$ subunit virus equivalent/well) were also found to be optimal for the detection of rabbit antibodies against SMV (Fig. 4).

3.4. Use of TSA to detect SMV-infected sheep

The serological response of lambs during an opportunistic SMV infection in our housed sheep flock is depicted in Fig. 5. The assay was carried out using TS antigen where the amount of subunit antigen in each well was equivalent to 250 $TCID_{50}$ virus. All lamb sera were assayed at a serum dilution of 1/200. It is evident that most of the lambs had sero-

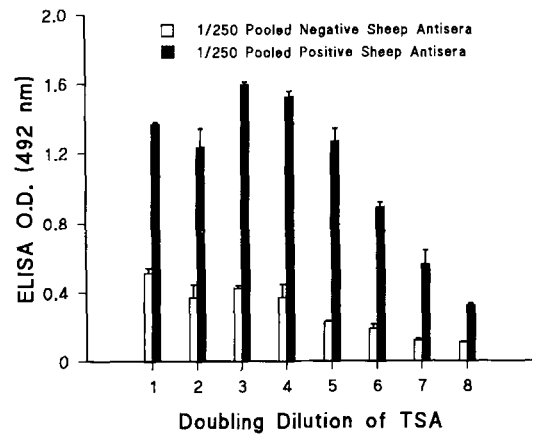

Fig. 3. Titration of total subunit SMV antigen as an ELISA reagent against sheep serum. Doubling dilutions of total subunit virus were coupled in triplicate to microtiter plates as described in Materials and Methods. Pooled negative (from naive sheep prior to vaccination) and positive (from SMV-hyperimmune sheep) sera were added at a final dilution of 1/250.

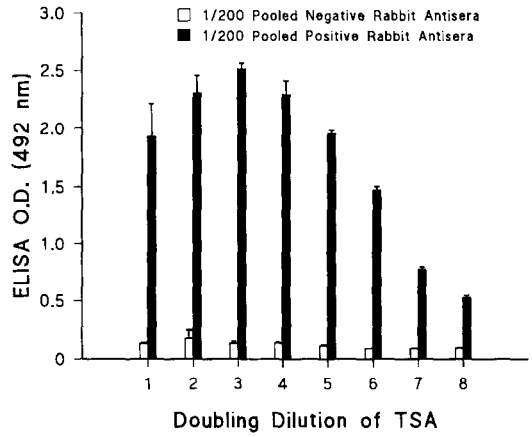

Fig. 4. Titration of total subunit SMV antigen as an ELISA reagent against rabbit serum. Doubling dilutions of total subunit virus were coupled in triplicate to microtiter plates as described in Materials and Methods. Pooled negative (from naive rabbits prior to vaccination) and positive (from SMV-hyperimmune rabbits) sera were added at a final dilution of 1/200.

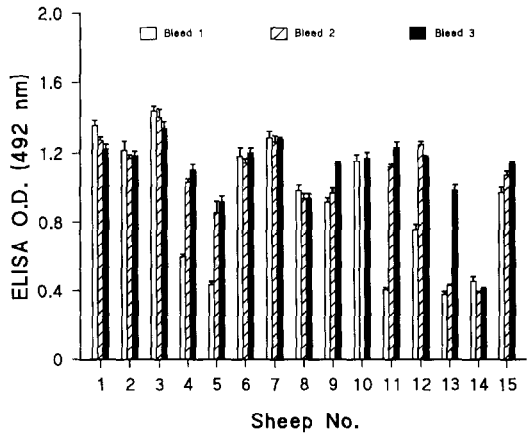

Fig. 5. ELISA reactivity against total subunit antigen of sera collected from lambs exposed to SMV. TSA was coupled to plates at a standard doubling dilution of 3. All lamb sera were assayed at a dilution of 1/250.

converted. Some animals eg. Nos 4, 5, 11 and 13 showed a sequential rise in ELISA reactivity but most of the lambs had high levels of reactivity against SMV at each of the 3 timepoints. Only animal No. 14 failed to show a rise in ELISA reactivity over time.

4. Discussion

The choice of an appropriate antigen is critical in determining the success of an ELISA test (Chin and Pang-Turner, 1990). Many commercially available assays for the detection of antibodies against viruses utilize inactivated virions as the assay reagent. Some of these assays require the use of blockers such as gelatin or skim milk to reduce the level of non-

specific background reactivity (Chin and Watts, 1992). As has been evident from this study, intact SMV virions do not provide a suitable assay reagent because comparatively large numbers of virus particles (2×10^4 $TCID_{50}$) are needed to generate a positive ELISA reading with an Optical Density (O.D.) of 0.8 when pooled positive hyperimmune serum was assayed at a dilution of 1/100. In contrast, subunit antigen generated from an equivalent amount of 500 $TCID_{50}$ virus provided an ELISA signal of > 1.6 O.D. at a serum dilution of 1/250. Clearly, subunit antigens provide a far more economical source of ELISA reagent to use than intact virions.

Conceptually, subunit antigens generated by disruption of the virus should provide an array of polypeptides for antibody binding. Intact virions on the other hand, would provide only a limited number of surface epitopes. The ELISA results confirm initial observations conducted to assess binding of serum antibodies from hyperimmune sheep with subunit antigens of SMV by immunoblotting (Fig. 1). This is the first report in the literature which documents the use of subunit antigens of SMV in an ELISA test. The relative ease of coupling diluted subunit antigens to microtiter plates would greatly facilitate detection and quantification of western blot-reactive antibodies that can otherwise, only be visualized by immunoblotting. Another advantage with the use of subunit antigen is the low background ELISA readings seen when pooled negative rabbit or sheep antisera were assayed. We have also found that it was not necessary when using subunit antigens to block non-specific binding sites on the plastic surface of each microtiter well with blockers.

Subunit antigens are generated easily with Laemmli's reducing mixture and unlike McKeever's procedure (McKeever et al., 1987), do not require a cold incubation step to precipitate SDS from the antigen extraction buffer. It is extremely likely that some antigens are lost during the cold precipitation step since proteins solubilised with negatively charged SDS may also co-precipitate with the SDS in the cold. The utility of subunit antigens to detect seroconversion is demonstrated in Fig. 5. It appears that most of the lambs had seroconverted and were positive reactors against SMV subunit antigens. We did not attempt in our assays to detect the level of maternal antibodies in the colostrum which could have contributed to the high levels of ELISA reactivity seen in first bleeds taken from most of the lambs. However, it would be possible to do this by carrying out an identical ELISA using a conjugated second antibody specific for sheep IgA.

Acknowledgements

The authors thank Roger Carrus and B. Turner for technical assistance. This work was supported by a grant from the Meat Research Corporation.

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