TOWARDS MOLECULAR DETECTION METHODS FOR APHID-BORNE STRAW-BERRY VIRUSES

C. D. Schoen and G. Leone
DLO Research Institute for Plant Protection (IPO-DLO)
Binnenhaven 5 P.O. Box 9060
6700 GW Wageningen The Netherlands

Abstract

Intact rhabdovirus particles of strawberry crinkle virus (SCV) mechanically transmitted from *Fragaria vesca* UC-S to *Physalis pubescens* plants were purified. When these particles were used as an immunogen, it was not possible to obtain either a virus-specific polyclonal antiserum after rabbit immunization or monoclonal antibodies. Each of the major nucleocapsid proteins (N, Ns and M) were therefore purified by means of preparative electrophoresis and injected into mice. The polyclonal mouse antisera raised showed a specific reaction with SCV-infected *P. pubescens* sag. Work is in progress to obtain specific monoclonal antibodies. It is also possible to isolate one ssRNA of about 13 kb from purified particles of SCV.

Relatively abundant dsRNA could be extracted from *Nicotiana occidentalis* 37B plants infected with strawberry mottle virus (SMoV). This dsRNA was composed of two bands of about 7.8 and 6.3 kbp which were DNase resistant. They became RNAse resistant in the presence of NaCI above 0.5 M. Work is in progress to make a cDNA library from purified dsRNA of SMoV. The need of replacing the leaf-graft indexing of strawberry for the major aphid-borne viruses in relation to the potential offered by the current knowledge on these viruses is discussed.

1. Introduction

The virus status of strawberry propagative material is still determined by indexing via leaflet grafts on sensitive *Fragaria vesca* clones for the major aphid-borne strawberry viruses, strawberry crinkle, strawberry mottle and strawberry mild-yellow edge virus (SCV, SMoV, SMYEV) (Anonymous, 1992). Although this detection method is time-consuming and inaccurate, it can index simultaneously for the three viruses. The development of rapid and reliable diagnostic methods based on serology for SCV, SMoV and SMYEV has been severely hampered until now by their unusual properties (Converse, 1992; Leone *et al.*, 1992). However, for SMYEV both molecular and serological detection methods seem to be applicable (Jelkmann *et al.*, 1989; Quail *et al.*, this volume; Jawee and Adams, this volume). As an indexing of strawberry based on different techniques is not desirable for practical applications, the potential new detection methods for SCV, SMoV and SMYEV should have a high degree of uniformity in order to replace completely the leaf-graft bioassay. Advances in the knowledge of the properties of each virus will help in finding the strategy for the development of these rapid, reliable and uniform detection methods that eventually can be adapted to routine indexing of strawberry propagative material.
In this paper we report some results of our efforts addressed to the development of detection methods for SCV and SMoV for the above mentioned purposes.

2. Materials and methods

2.1. Maintenance of SCV and SMoV and passage to herbaceous hosts

Isolate Heidelberg of SCV was maintained in *F. vesca* UC-5 plants, transferred to *Nicotiana occidentalis* subsp. *obliqua* Pl by aphids (*Chaetosyphon fragaefolii*) and propagated in the latter plant in a temperature-controlled greenhouse at 20°C, with additional illumination during winter to make a total daylength of 16 hr. The virus was transferred weekly to *Physalis pubescens* seedlings having three to four true leaves. Inoculum was prepared by grinding 50 leaf disks (0.5 cm in diameter) of *N. occidentalis* subsp. *obliqua* Pl, carrying local lesions, in 5 ml of a 1% nicotine solution. The slurry was immediately rubbed onto carborundum-dusted leaves.

Isolate 1134 of SMoV was maintained in *F. vesca* UC-5, transmitted and propagated in *N. occidentalis* 37B, as described by Leone *et al.* (this volume).

2.2. Purification of SCV

Symptomatic leaves of *P. pubescens* were harvested 18-25 days after inoculation, stored at 4°C and extracted as described previously (Leone *et al.*, 1992). After ratezonal sedimentation on a preformed 10-40% sucrose gradient, the opaque, diffuse band containing SCV particles was collected and the virus pelleted by high speed centrifugation at 35,000 rpm in a Ti 60 Beckman rotor for 1 h. For intact virus particle purification, the pellet obtained was subjected to CsCI (32 mg/ml) equilibrium centrifugation in 10% sucrose and 10 mM EDTA. After a 16 h centrifugation step at 30,000 rpm in a SW 41 Beckman rotor, a sharp virus band was collected and extensively dialysed against 10 mM Tris-HCI, pH 7.8, before any further use. For nucleocapsid purification, the pellet was resuspended in 0.01 M Tris-HCI pH 7.6, containing 0.01 M sodium sulfite, 0.01 M EDTA, and 1% Nonidet P40. After clarification by low-speed centrifugation at 8,000 g for 10 min, the supernatant was centrifuged on a 30% sucrose cushion for 1 h at 125,000 g. The pellet was resuspended in 0.01 M Tris-HCI, pH 7.6 and used for further studies.

2.3. Polyacrylamide gel electrophoresis of SCV structural proteins

Preparations of intact SCV were disrupted in 62.5 mM Tris-HCI, pH 6.8, 3% SDS, 1% β-mercaptoethanol, boiled for 4 min at 95°C and applied to 12.5% polyacrylamide-SDS minigels. Electrophoresis was carried out using the SDS discontinuous buffer system of Laemmli (1970) in a Mini-Protean II dual slab cell (Bio-Rad). For molecular weight estimates, low range SDS-PAGE standards (Bio-Rad) were used. Proteins were stained either with Coomassie Brilliant Blue or with the Bio-Rad Silver Stain Plus Kit.

2.4. Purification of the M, N and N nucleocapsid proteins of SCV

The different nucleocapsid proteins of SCV were each purified by preparative SDS-PAGE using the Bio-Rad Model 491 Prep Cell. As for the analytical SDS-gel electrophoresis, the discontinuous buffer system of Laemmli (1970) was employed. A 6 cm high 12.5% polyacrylamide separating gel was polymerized in the tube (3.7 cm in diameter) of the preparative gel apparatus. Thereafter, a 2 cm high 3% polyacrylamide separating gel was polymerized on top of the separating gel. The virus preparation, containing approximately 1.5 mg of protein, was dissolved in 2 ml of SDS sample buffer (42 mM Tris-HCl, pH 6.8, 8% glycerol, 3% β-mercaptoethanol, 0.1% SDS, 0.01% bromophenol blue) and incubated at 95°C for 5 min. The sample

was then loaded and the gel was run for about 8 h at 70 mA constant current until the bromophenol blue reached the bottom of the separating gel. The power supply was adjusted to 50 mA constant current and SDS running buffer (50 mM Tris, 384 mM glycine, 0.1% SDS) was pumped through the elution chamber at a flow rate of 1 ml/min. When the bromophenol blue started to elute out of the separating gel, fractions of 3 ml each were collected. Capsid proteins were located by analytical SDS-PAGE. Fractions containing the protein of interest were pooled and freeze-dried. For antiserum production, the SDS was removed by precipitation, titrating the solution with KCl in the presence of 0.3% Nonidet P40. Protein concentration was estimated using the Pierce BCA Protein Assay.

2.5 Preparation of polyclonal antiserum

Antiserum was produced by injecting intraperitoneally 30 µg of purified SCV capsid protein in Freunds complete adjuvant into female Balb/c mice followed by two injections of 30 µg capsid protein mixed with incomplete Freunds adjuvant at monthly intervals. Samples of serum obtained from the tails of the mice were assayed for the presence of antibodies against SCV by indirect antigen coated plate-ELISA (IACP-ELISA) and by Western immunoblotting.

For IACP-ELISA, plates were coated for 2 hr with 1 µg/ml of infected or non infected leaf material at 37°C and then blocked for 30 min with PBS (188 mM NaCI, 3 mM KCI, 10 mM Na_2HP0_4, 3 mM KH_2P0_4), 0.2% Tween 20 and 5% dried milk powder. Series of dilutions of the antisera were incubated in the plates for 2 hr at 37°C. Then rat anti-mouse alkaline phosphatase conjugate (Gibco-BRL) was added at the manufacturer's recommended concentration and incubated for 2 hr at 37°C. After the addition of the substrate p-nitrophenyl phosphate (Sigma) at 0.5 mg/ml in 10% (v/v) diethanolamine, pH 9.8, the absorbance of each well was read at 405 nm (A_{405}) after 1 hr at 37°C on a ELISA plate reader.

For Western immunoblotting, proteins were transferred electrophoretically from SDS-polyacrylamide gels to nitrocellulose membranes (0.45 µm pore size) at 100 Volts for 1 hr in 25 mM Tris, 192 mM glycine, 20% methanol using a Mini Trans-Blot apparatus (Bio-Rad). The blots were cut along lanes with prestained molecular weight markers (BioRad) and blocked with 1% BSA, and 0.05% Tween 20 in PBS (blocking buffer) overnight at 4°C. The primary antibodies were diluted in blocking buffer and incubated 2 hr at room temperature. After three washes in PBS at room temperature for 15 min each, the nitrocellulose was incubated with rat anti-mouse antibody conjugated with alkaline phosphatase (Gibco-BRL), also diluted in blocking buffer. After four washes with PBS with 0.05% Tween 20, the color reaction was performed with the bromo- chloro-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate as described by Harlow and Lane (1988).

2.6 Purification of RNA from SCV

RNA was extracted from the intact virus particles or from the nucleocapsid after adding SDS to a final concentration of 1%, (w/v) followed by phenol and subsequent phenol/chloroform (1:1) extractions. After centrifugation at 10,000g, the RNA was precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.4) and 2.25 volumes of absolute ethanol. The RNA was then stored at -20°C overnight. The RNA was pelleted by centrifugation at 10,000 g, and the supernatant was poured off. The pellet was washed in 70% cold ethanol and dried under vacuum. The RNA

was resuspended in sterile deionized water and electroforezed in the presence of methylmercuric hydroxide on a 1% agarose gel. Bands were visualized by UV light after incubation of the gel for 15 min in 0.5 M ammonium acetate in the presence of 0.5 µg/ml EtBr.

2.7. Purification and characterization of dsRNA of SMoV

Double-stranded RNA was isolated from SMoV (isolate 1134)-infected *N. occidentalis* 37B plants according to the procedure described by Jordan (1986) with minor modifications. Samples were electrophorezed in 0.75% agarose slab gels for 2 h at 75 V in 40 mM Tris, 40 mM sodium acetate, 1 mM EDTA (TAE buffer), pH 8.0. EtBr was incorporated in the gels at a concentration of 0.5 µg/ml and bands were visualized by UV light. Identification of dsRNA was accomplished by DNase I-RNAse A_1 treatments of the samples prior to electrophoresis, essentially as reported by Jordan (1986).

2.8. cDNA synthesis

Double-stranded RNA of SMoV was denatured by heating at 65°C for 10 min just before cDNA synthesis. Synthesis of cDNA from dsRNA template was carried out using the TimeSaver™ cDNA synthesis kit provided by Pharmacia using a hexadeoxynucleotide ($pd(N)_6$) as a random reverse transcriptase primer. cDNA was cloned into the lambda ExCell EcoR I/CIP cloning vector from Pharmacia. *In vitro* Lambda packaging of the recombinant lambda DNA was performed with the Ready.To.Go Lambda Packaging Kit (Pharmacia). Plasmid DNA's were extracted as recommended (Promega, Madison, WI 53711, USA). Unless otherwise stated, molecular biology techniques were performed as described in Sambrook *et al.* (1989).

3. Results

3.1. Preparation of polyclonal antisera against SCV

Virus purifications from *P. pubescens* plants were performed to obtain intact rhabdovirus particles for the development of monoclonal antibodies in mouse and polyclonal antiserum in rabbit (results not shown). All the antisera raised against intact SCV particles were not specific for infected plant material.

In order to improve the development of specific antisera, the nucleocapsid proteins (N, N_S and M) were singly purified by means of preparative electrophoresis (Fig. 1). The M protein was injected directly into a mouse while the N and the N_S proteins were mixed before injections. Table 1 shows the response of a polyclonal antiserum raised in mouse, measured in an indirect coated plate-ELISA (IACP-ELISA) assay. A clear difference between infected and healthy *P. pubescens* leaf material was observed, particularly with the antiserum raised against the $N+N_S$ proteins. These results were confirmed also by Western immunoblotting (Fig. 2).

3.2. Isolation and purification of ssRNA of SCV

Purified intact particles or nucleocapsid preparations were used for the isolation of ssRNA. RNA extractions from capsid preparations yielded a high amount of RNA of SCV (ca 13 kb) but other RNA contaminants were also present (Fig. 3). More pure RNA, although in lower amount, was obtained when intact SCV particles underwent CsCl equilibrium centrifugation in 10% sucrose and 10 mM EDTA.

3.3. Isolation, characterization and molecular cloning of dsRNA of SMoV

Two bands of about 6.3 and 7.8 kbp were found on agarose gel after traditional phenol extraction and affinity chromatography on Whatman CFll cellulose powder of *N. occidentalis* 37B plant material infected with SMoV. These two bands were not present in healthy plants. Their dsRNA nature was determined by their resistance to DNase (Fig. 4). Both bands became RNase resistant in the presence of NaCI above 0.5 M (Fig. 5).

Molecular cloning of the purified dsRNA bands resulted in the isolation of 22 clones containing cDNA fragments. These clones are currently used for further characterization.

4. Discussion

SCV was identified as a rhabdovirus two decades ago (Richardson *et al.*, 1972) but its successful purification and partial characterization has been reported only recently (Hunter *et al.*, 1990; Leone *et al.*, 1992). Although Hunter *et al.* (1990) succeeded in preparing an antiserum against the G protein of SCV, this could not be applied as a diagnostic tool to replace the routine detection of strawberry propagative material by grafting to indicator plants. In our experience, polyclonal antisera from rabbit and mouse raised against intact SCV particles were also not specific for infected plant material. This is probably due to the low immunogenicity of SCV, a property shared by other rhabdoviruses (Jackson *et al.*, 1987). However, polyclonal mouse antisera raised against the M protein and the N plus N_S proteins, purified by preparative electrophoresis, showed a specific reaction with SCV infected *P. pubescens* material in both IACP-ELISA and Western blotting assay. Work is in progress to obtain monoclonal antibodies against the N, N_S and M proteins of SCV as well as the cloning of its RNA.

The dsRNA nature of the two bands associated with SMoV infected *N. occidentalis* 37B described by Leone *et al.* (this volume), has been established. This dsRNA has the uncommon property of being RNase A_1 resistant only in the presence of NaCl above 0.5 M, while normally almost complete resistance is achieved at 0.2-0.3 M NaCI (Sorrentino *et al.*, 1980). We started to clone this dsRNA of SMoV in order to obtain cDNA probes suitable for virus detection.

In conclusion, while for SCV it seems possible to develop both a serological and a molecular biological based detection method, for SMoV only the last possibility seems plausible because of its defective properties (Leone *et al.*, this volume). The replacement of the current method of indexing strawberry by grafting on sensitive indicators for the major aphid-borne strawberry viruses SCV, SMoV and SMYEV with rapid, specific and sensitive detection methods needs a high level of uniformity for the new potential methods, in order to be competitive with the bioassay. This is necessary because an indexing based on different techniques is not desirable for practical applications when the current one can index simultaneously SCV, SMoV and SMYEV. As for SMYEV, primers and sequence have already been published (Hadidi *et al.*, 1993; Jelkmann *et al.*, 1992), it is anticipated that the development of diagnostic tools for the simultaneous detection of SCV, SMoV and SMYEV will especially rely on molecular biological methods.

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Table 1 IACP-ELISA assay of polyclonal antisera raised in mouse against the major nucleocapsid proteins of SCV.

Antisera	*P. pubescens*	Antisera dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
pre-immune serum	infected	0.087[a]	0.044	0.035	0.035	0.035
M	healthy	0.110	0.067	0.038	0.028	0.025
M	infected	0.385	0.167	0.054	0.040	0.035
$N+N_S$	healthy	0.115	0.087	0.075	0.045	0.025
$N+N_S$	nfected	0.467	0.490	0.317	0.158	0.066

a Absorbance measured at 405 nm

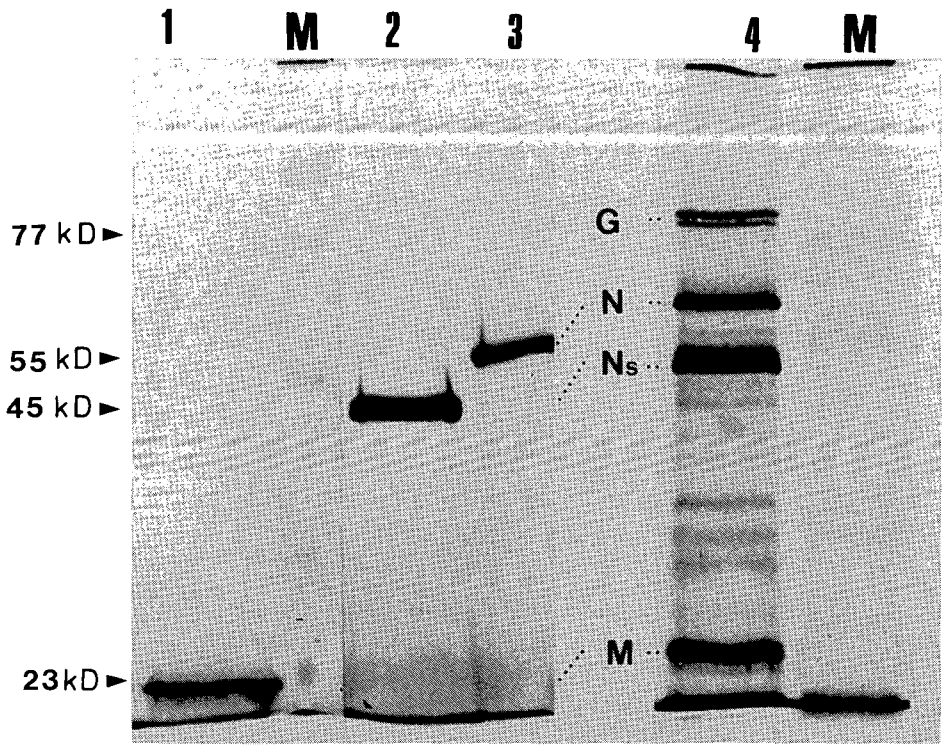

Figure 1 - Electrophoresis of the major nucleocapsid proteins of SCV, singly purified by preparative SDS-PAGE. Lane 1, M protein; lane M, molecular-weight markers; lane 2, N_S protein; lane 3, N protein; lane 4, SCV structural proteins after CsCl-equilibrium centrifugation; lane M, molecular-weight markers.

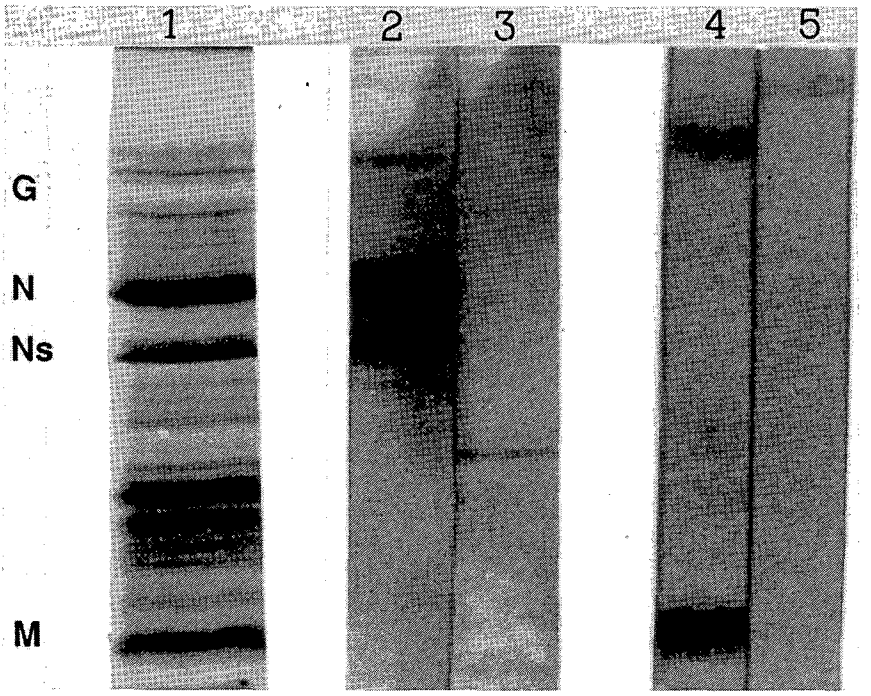

Figure 2 - SDS-PAGE followed by Western immunoblotting of partially purified *P. pubescens* material infected with SCV. Bands were visualized by means of: Lane 1, Coomassie Brilliant Blue; lane 2, antiserum against the $N+N_S$ proteins; lane 3, pre-immune serum; lane 4, antiserum against the M protein; lane 4, pre-immune serum.

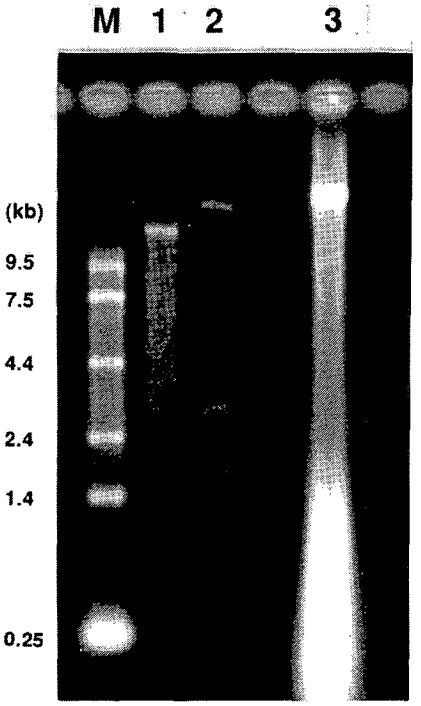

Figure 3 - Agarose gel electrophoresis of RNA of SCV. Lane M, molecular-weight markers; lane 1, RNA of a rhabdovirus in *Alstroemeria caryophylla*; lane 2, RNA of SCV extracted from intact virus particles after CsCl-equilibrium centrifugation; lane 3, RNA of SCV extracted from nucleocapsid preparations.

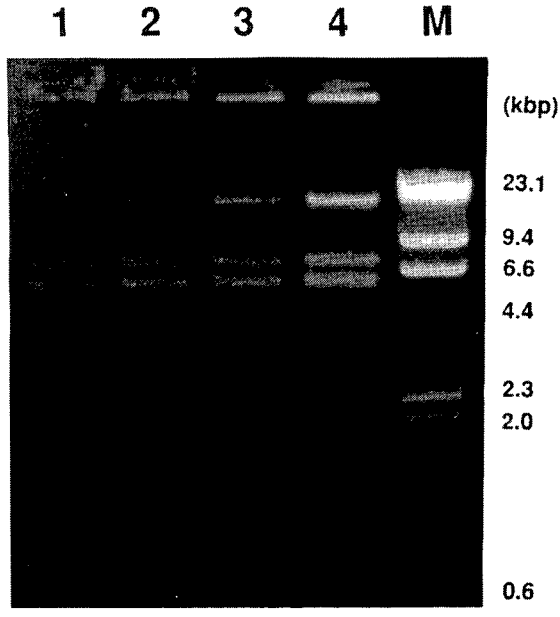

Figure 4 - DNase treatment (end concentration of DNase I: 70 µg/ml) of dsRNA of SMoV isolated from *N. occidentalis* 37B. Lane 1, dsRNA after a 20 min treatment at 37°C; lane 2, dsRNA after a 5 min treatment at 37°C; lane 3, dsRNA after a 2 min treatment at 37°C; lane 4, non-treated sample; lane M, λ-Hind III markers.

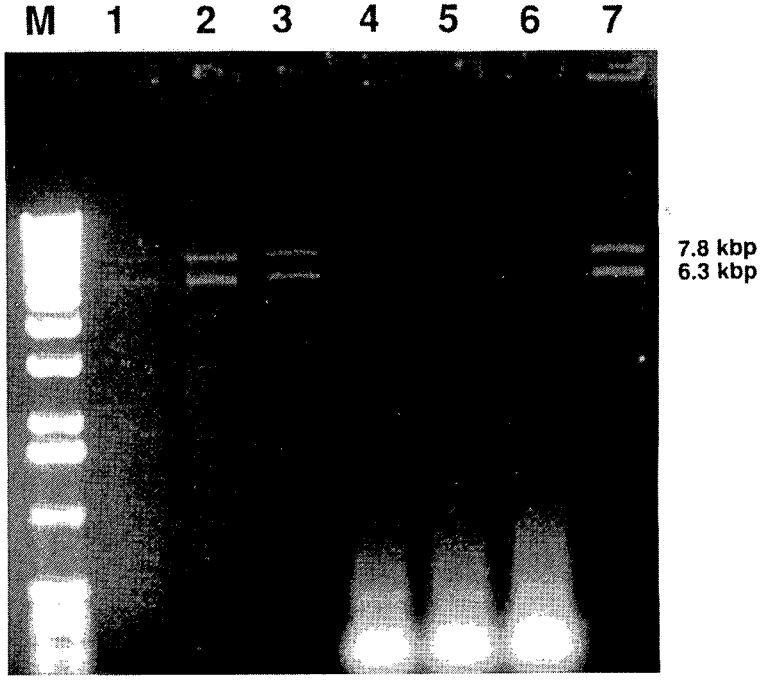

Figure 5 - RNase treatment (end concentration of RNase A_1: 5 µg/ml) of dsRNA of SMoV isolated from *N. occidentalis* 37B. Treatments occurred at 37°C for 20 min. Lane M, DNA markers (1 kb); lane 1, dsRNA after digestion in 0.3 M NaCl; lane 2, dsRNA after digestion in 0.5 M NaCl; lane 3, dsRNA after digestion in 0.7 M NaCl; lane 4, RNA markers (1 kb) after digestion in 0.3 M NaCl; lane 5, RNA markers after digestion in 0.5 M NaCl; lane 6, RNA markers after digestion in 0.7 M NaCl; lane 7, non-treated dsRNA.