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Comparison of Three Methods for Assessing Plum Pox Virus Variability: Further Evidence for the Existence of Two Major Groups of Isolates

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With 3 figures

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Abstract

Twenty-eight plum pox virus isolates from several European and Mediterranean countries were compared by electrophoretic mobility of their coat protein subunit measured by Electroblot Immuno-Assay (EBIA), antigenic properties of the N- and C-terminal parts of the coat protein and restriction fragment length polymorphism (RFLP) and analysis of polymerase chain reaction (PCR) amplification of the C-terminal part of the coat protein gene. Similar results were obtained by each of the three methods. These confirm the existence of the two major subgroups of PPV, and which we now propose to designate PPV-D and PPV-M, respectively.

Zusammenfassung

Vergleich dreier Methoden verwendet zur Schätzung der Variabilität von Plum Pox Virus: Zusächliche Evidenz für das Bestehen von zwei Hauptgruppen von Isolaten

28 PPV Isolate und Stämme aus europäischer und mittelländlischer Herkunft wurden durch drei verschiedene Methoden verglichen Elektrophoretische Mobilität der Hüllprotein-Untereinheit, gemessen durch ElectroBlot Immuno-Assay (EBIA); antigenische Eigenschaften der N und C-Endteile des Hüllproteins, und RFLP Analyse eines PCR Fragments entsprechend des C-Endteiles des Hüllprotein-Gens.

Es wurde eine vollständige Korrelation der Ergebnisse von den drei Methoden beobachtet. Dieses Ergebnis bestätigt durchaus die Existenz der zwei überwiegenden Untergruppen von PPV. und es wird hier vorgeschlagen, diese zwei Gruppen PPV-D und PPV-M zu nennen.

Plum pox is now the most severe and economically important virus disease of stone fruit trees (*Prunus sp.*), and plum pox potyvirus (PPV) continues to cause problems for European fruit growers. The use of efficient methods which allow discrimination of viral populations would greatly facilitate the understanding of PPV epidemiology and in defining control strategies.

The first conclusive serological discrimination of PPV isolates was reported by KERLAN

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and DUNEZ (1979), who described two serotypes (D and M). Recently, other methods have also provided some information about the genetic diversity of PPV. VARVERI *et al.* (1988) developed an RNA probe covering the carboxy-terminal half of the protein gene of PPV isolate Dideron (PPV-D) which produced a weak differential response when reacted with the Markus (PPV-M) and homologous isolates. WETZEL *et al.* (1990) selected probes representing non-structural viral protein genes which were equally sensitive in detecting both PPV-D and PPV-M strains.

Recently, the polymerase chain reaction (PCR), a method more sensitive than molecular hybridization, has been developed for the detection of PPV (KORSCINECK *et al.* 1991, WETZEL *et al.* 1991a, 1992). A restriction site polymorphism in the amplified fragments differentiated two groups of isolates, but a clear correlation between the restriction profile and the serological classification was not then established (WETZEL *et al.* 1991a).

Results published in recent years have provided abundant information about the structure and expression of the PPV genome. Thus, complete genome sequences have been determined for PPV-D (TEYCHENEY *et al.* 1989), PPV-NAT (MAISS *et al.* 1989) and PPV-R (LAIN *et al.* 1989). These show levels of identity in excess of 98%. A fourth isolate (PPV-E1-Amar) has been partially sequenced, and shown to have 80% identity with PPV-D, PPV-NAT and PPV-R (WETZEL *et al.* 1991b). More recently, CERVERA *et al.* (1993) described the 3-terminal sequence of two other PPV isolates (PPV-PS and PPV-06) and showed that both differed from strains sequenced previously.

In addition to the analysis of nucleotide sequences, serological analysis of the N- and C-terminal parts of the coat protein, as used for other potyviruses (SHUKLA *et al.* 1988, 1989), offers another possibility of evaluating the relationships between different PPV isolates.

The purpose of this study was to evaluate and compare the following three techniques for the analysis of the variability of a wide range of PPV isolates from several countries: electrophoretic mobility of the coat protein subunit, immunological properties of N and C-terminal parts of coat protein, and restriction site polymorphism in the coat protein gene following PCR amplification.

Materials and Methods

Strains and isolates of PPV

Twenty-eight strains or isolates of PPV were collected or obtained from European and Mediterranean countries (Table 1). They were usually propagated in seedlings of GF305 peach and *Pisum sativum* L. cv. Colmo. The plants were maintained in separate cabinets in an insect-proof glasshouse to avoid cross contamination.

Differentiation of isolates using antibodies directed towards N- and C-terminal parts of the coat protein

Virus purification

PPV was purified from *P. sativum*, using a method described by GOUGH and SHUKLA (1981), but slightly modified as follows: PPV was extracted by grinding plant tissue in one and a half volumes of 0.1 M sodium borate at pH 8.0, containing 0.15% thioglycollic acid and 0.01 M EDTA, and one-half volume each of chloroform and carbon tetrachloride. Three cycles of differential centrifugation (8000 $\times$ g for 10 min and 100 000 $\times$ g for 90 min) were followed by ultracentrifugation (208 000 $\times$ g for 120 min) through a linear 10–40% sucrose gradient in 0.02 M sodium borate buffer pH 8.0. The virus was finally concentrated by ultracentrifugation at 100 000 $\times$ g for 90 min.

Table 1
PPV isolates and strains used in this study

Isolate	Origin			
	Country	Host	Supplier	Year[1]
F117	France	apricot	M.Boeglin (INRA–Montpellier)	1991
Dideron	France	apricot	J. Dunez (INRA–Bordeaux)	1992
F97	France	peach	S.P.V.	1991
F116	France	apricot	G. Labonne (INRA–Montpellier)	1985
F118	France	apricot	S.P.V.	1991
F63	France	apricot	J.B. Quiot (INRA–Montpellier)	1985
F96	France	peach	S.P.V.	1991
F119	France	apricot	S.P.V.	1991
F14	France	peach	G. Labonne (INRA–Montpellier)	1989
F66	France	apricot	J.B. Quiot	1991
F67	France	apricot	J.B. Quiot	1985
F24	France	peach	M. Boeglin	1992
F120	France	peach	M. Boeglin	1991
Y2744	Yugoslavia	plum	C.T.I.F.L.	1991
R4776	Romania	—	C.T.I.F.L.	1985
G57	Germany	plum	S.P.V.	1992
A58	Austria	—	F. Dosba (INRA–Bordeaux)	1992
A123	Austria	—	F. Dosba	1992
Y100	Yugoslavia	peach	F. Dosba	1991
EL-AMAR	Egypt	apricot	J. Dunez	1987
MARKUS	Greece	peach	F. Leclant (INRA–Montepellier)	1987
B71	Bulgaria	plum	F. Leclant	1987
G98	Germany	plum	S.P.V.	1991
S72	Spain	plum	G. Labonne	1991
Y101	Yugoslavia	apricot	F. Dosba	1992
Y56	Yugoslavia	plum	F. Dosba	1992
T60	Turkey	apricot	F. Dosba	1992
R59	Russia	plum	F. Dosba	1992

[1] Year of introduction to our collection.
S.P.V.: Service de la Protection des Vegetaux.
C.T.I.F.L.: Centre Technique Interprofessional des Fruits et Légumes.

Removal of N- and C-terminal peptides of the coat protein from assembled particles

Freshly purified virus (10 mg) was incubated with trypsin (Sigma type XIII, TPCK treated) using 6 μg of enzyme per mg of virus for 30 min at 25°C (the enzyme-virus ratio was chosen to selectively remove the N- and C-terminal peptides regions of coat protein as verified by EBIA). The enzyme-resistant core particles were separated from the N- and C-terminal peptides by centrifugation at 126000 $\times$ *g* for 90 min at 4°C.

Selection of N- and C-terminal specific IgG

The pellet containing the trypsin-resistant core particles was resuspended in borate buffer (0.02 M, pH 8) and dissociated as described by SHUKLA *et al.* (1988). The protein preparation was coupled to 1 g CNBr sepharose 4B (Sigma) according to the manufacturer's instructions. One hundred μl of

IgG (1 mg/ml) directed towards intact particles of PPV-D (kindly provided by C. VARVERI, INRA-Bordeaux) were loaded on to the column in 20 ml phosphate saline buffer and the unbound IgGs were collected.

Electroblot immuno-assay (EBIA)

EBIA was performed using the method previously used for potyviruses (QUIOT-DOUINE *et al.* 1990). Proteins from 10-fold diluted plant sap extracts (EHLERS *et al.* 1986) or from purified viral preparations (15 μg) were first separated by electrophoresis on discontinuous denaturing 7.5–15% polyacrylamide gradient gels (LAEMMLI, 1970). Electrophoresis was carried out in a mini-gel apparatus, proteins were then electro-transferred onto nitrocellulose membranes as described by TOWBIN *et al.* (1979) and detected immunologically. The antisera from goats were either directed towards intact particles or towards N- and C-terminal peptide regions of the coat protein of PPV-D. Bound antibodies were visualised using a rabbit anti-goat IgG-horseradish peroxidase (HRP) conjugate and HRP colour development reagents (Biorad).

Immunocapture — PCR (IC/PCR) and restriction site analysis

The immunocapture-PCR was performed according to the protocol of JANSEN *et al.* (1990) adapted for PPV by WETZEL *et al.* (1992) as follows: sterile Eppendorf tubes were coated with anti PPV-D IgG (2 μg/ml) using infected plant extracts as the antigen source. Triton X-100 solution (1%) heated to 65°C was added to release the viral RNA; chemical denaturation, reverse transcription of the viral RNA and amplification of the synthetised cDNA were as described by WETZEL *et al.* (1991a).

The region of the PPV genome selected for amplification is of 243 bp encoding the carboxyl terminus of the coat protein. The pair of oligonucleotide primers used were described by WETZEL *et al.* (1991a): 5′ACCGAGACCACTACACTCCC3′ (sense primer), and 5′CAGACTACAGCCTCG-CCAGA3′ (antisense primer).

*Rsa*I restriction digestions were effected using 5 U of enzyme with 10 μl aliquots of the amplified fragments. The digestion was carried out at 37°C for 2 h in the buffer supplied by the enzyme manufacturer. The product was analysed by electrophoresis on a 6% polyacrylamide gel in Tris-borate-EDTA buffer (MANIATIS *et al.* 1982), and bands were visualized by ethidium bromide staining.

Results

Grouping isolates by their electrophoretic properties

The coat protein subunits of all PPV isolates were easily detected in EBIA using an antiserum to intact particles of PPV-D (Fig. 1). Two groups were distinguished on the basis of electrophoretic mobility; the first group of isolates (lanes 4 to 7), including isolates homologous to the antiserum (PPV-D) and corresponding to the D serotype described by KERLAN and DUNEZ (1979), reacted strongly. The coat protein subunits were estimated to have molecular weights of 36 kDa. Slight differences in mobility were observed within the two groups but their extents were too weak to be significant. Partial degradation of the coat protein was observed with all the isolates tested.

The relative electrophoretic mobility of each group was reproducible when the experiment was performed with extracts from different plant sources such as sweet pea, GF 305 peach seedlings, peach and apricot. Similar results were obtained using anti-M serotype sera prepared in our laboratory to PPV-14 or in Bordeaux to PPV-M.

Thus, coat protein electrophoretic mobility confirms the separation of PPV isolates into two major groups, and the names proposed for these are D and M with reference to the serotypes described by KERLAN and DUNEZ (1979).

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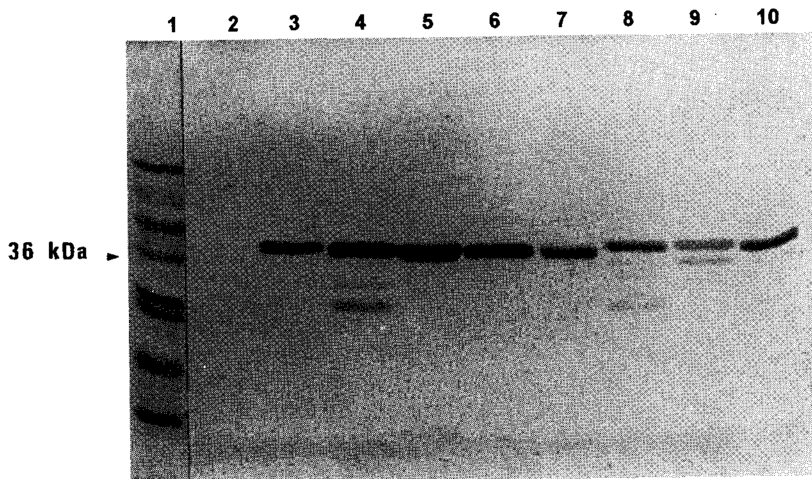


Fig. 1. Detection of 8 PPV isolates in plant sap by electroblot-immunoassay using an anti PPV-D serum prepared against intact particles. Lane (1): low molecular weight standards (Sigma) (2): healthy plant (3): PPV-Y2744 (4): PPV-R4776 (5): PPV-G57 (6): PPV-A58 (7): PPV-A123 (8): PPV-Y100 (9): PPV-El-Amar (10): PPV-B71, respectively.

Grouping isolates according to the antigenic properties of the N- and C-parts of their coat proteins

The original unfractionated polyclonal PPV-D antiserum reacted with both group D and group M isolates; antibodies directed to the N- and the C-termini of the PPV-D coat protein, selected by affinity chromatography as described in Materials and Methods, were found to be highly specific for those of group D. As shown in Fig. 2, they reacted strongly to group D isolates (lanes 2, 4, 5 and 7) but showed no cross reaction with those of group M (lane 3 and 6). The absence of reactions with the core protein of PPV-D shows the specificity of the selected IgG (lane 8).

In addition, the electrophoretic mobility typing data and the results obtained by this second technique were closely correlated. Thus, analysis of the serological properties of the N- and C-terminal variable regions of the coat protein confirms the segregation of PPV-D isolates into D and M serotypes.

Grouping isolates by RFLP Analysis of an amplified cDNA fragment corresponding to the 3′ end coat protein gene

After IC/PCR amplification, all isolates produced a single band of the expected size (243 bp), whereas preparations from the healthy plant control produced no band.

The restriction analysis of the amplified fragments showed the presence of an *Rsa*I restriction site in all group D isolates (Fig. 3, lanes 2, 9, 11, 12 and 14). As expected from the sequence of PPV-D (TEYCHENEY *et al.* 1989), the *Rsa*1 cleavage resulted in the appearance of two DNA products with approximate sizes of 162 and 81 bp. A similar pattern was observed for all the serotype D isolates. Conversely, no *Rsa*1 digestion was observed with any of the serotype M isolates tested (Fig. 3, lanes 3, 4, 5, 6, 7, 8, 10 and 13). Again, the typing obtained with this technique correlated perfectly with that obtained by electrophoretic mobility or serological typing of the N- and C-terminal fragments.

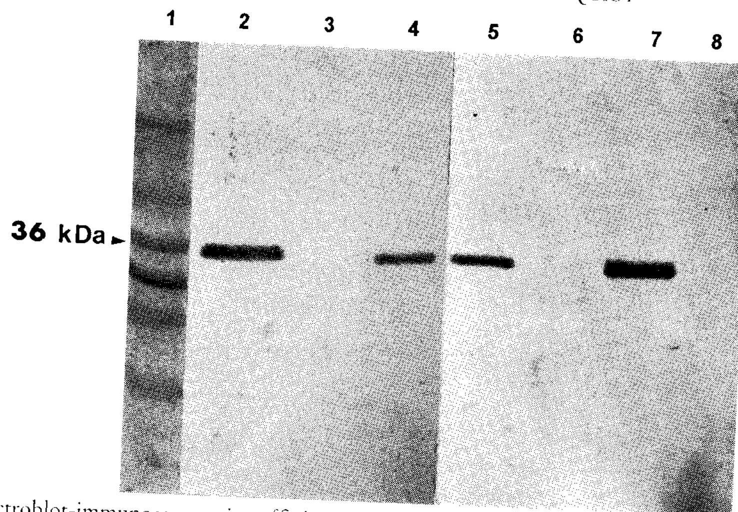


Fig. 2. Electroblot-immunoassay using affinity-selected IgG directed to N- and C-termini of PPV-D coat protein. Lane (1): low molecular weight standards (Sigma), (2) to (6): sap extracts from plants infected with, respectively, (2): PPV-D (3): PPV-F14 (4): PPV-F116 (5): PPV-S72 (6): PPV-El-Amar. Lane (7): untreated purified PPV-D (8): trypsin resistant core protein of PPV-D.

Discussion

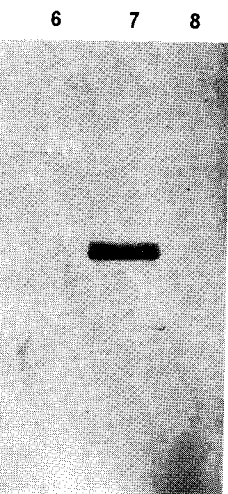
Twenty-eight different PPV isolates from different geographical origins and species of source plant, were compared by three different methods. Two methods permitted the classification of the isolates into two groups, i.e. according to the occurrence of a restriction site near the 3′ terminus in the case of the PCR method or to the presence or absence of serological cross reactions between the N- and the C-terminal variable parts of the coat protein. The third method, based on differences in electrophoretic mobility of the coat protein, also finely discriminates isolates within the two basic groups.

The three procedures were effective with all the isolates and, to our surprise, showed a close correlation in the typing of the 28 isolates (Table 2). Our results thus strongly support the separation of PPV isolates into two groups.

Furthermore, because each group shares several specific properties located on different parts of the coat protein, they probably correspond to the two forms which are best fitted for survival under natural conditions. Our results show that such PPV types occur in several European countries and can be recovered from naturally infected fruit trees as well as from graft-inoculated peaches (GF 305) or from mechanically inoculated peas. This suggests that isolates of these two groups were not selected in the laboratory and that they correspond to naturally occurring PPV field populations.

The use of SDS-PAGE to separate proteins from crude extracts in minigels, followed by blotting into nitrocellulose sheets, is a rapid method for immunodetection and classification of PPV isolates. Unfractionated antisera are suitable for the detection of both groups, and we were able to differentiate isolates by their electrophoretic mobility properties. Direct serological differentiation was possible using IgG directed to the N- and C-terminal regions of the coat protein and selected by immunoaffinity chromatography from polyclonal antisera prepared against the whole particle.

The sensitivity of RT/PCR as a detection method for PPV was evaluated initially by KORSCHINECK *et al.* (1991) and WETZEL *et al.* (1991a, 1992). Using a limited number of



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6 (5): PPV-S72 (6): PPV-El-Amar.
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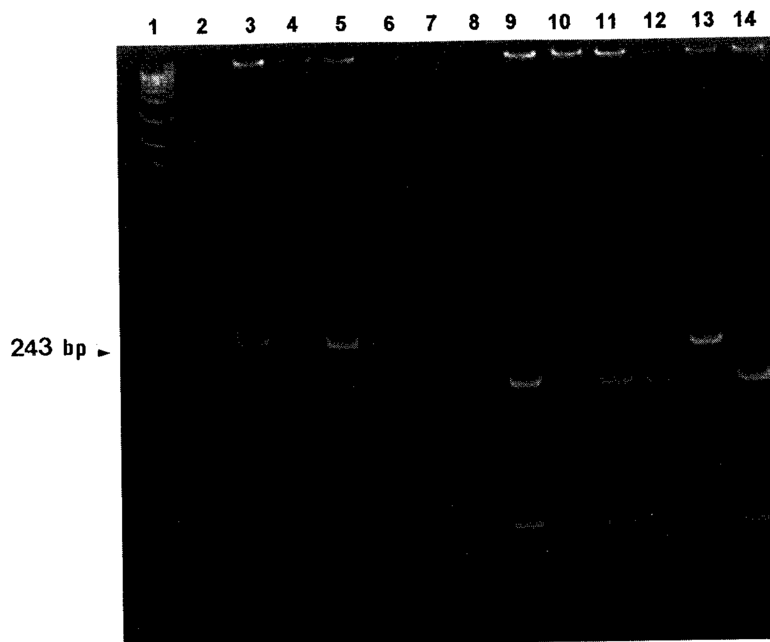

Fig. 3. RsaI restriction site analysis of the IC/PCR-amplified fragments from 13 PPV isolates. Lane (1): molecular weight standards (123 DNA Ladder, B.R.L.): lanes (2)-(14): digested fragments of PPV isolates: Dideron, El-Amar, B71, F14, F14 (Bordeaux's collection) F24, F97, S72, Y100, G98, Y56, T60, A58, respectively. After amplification and restriction enzyme digestion, fragments were separated by electrophoresis on 6% polyacrylamide gel and photographed under UV light after ethidium bromide staining.

isolates, WETZEL *et al.* (1991a) showed that a unique *Rsa*1 site was polymorphic in the amplified fragments. Using a much larger number of isolates, we have confirmed and extended their results by establishing a correlation between the *Rsa*1 restriction site polymorphism and the two groups established by electrophoretique mobility criteria and immunological properties of N- and C-terminal parts of coat protein. Thus, the presence of *Rsa*1 site assigns an isolate to the D group. An artificial mixture of the two types of isolates was readily distinguishable using RFLP, both digested and undigested bands being apparent after electrophoresis (data not shown).

A similar relationship between serological and sequence properties has been observed for cucumber mosaic virus (CMV); thus, the two CMV serotypes described by DEVERGNE and CARDIN (1973) are correlated with restriction site polymorphism and sequence divergence (RIZOS *et al.* 1992). The data suggest that such a convergence of properties can be observed in at least two widely different groups of plant viruses.

According to SHUKLA *et al.* (1988) and SHUKLA and WARD (1989a), the N-terminus is the most immunodominant region of the coat protein of potyviruses, and antibodies generated towards this region are highly specific and recognize only the homologous viruses and their strains. By this approach and on the basis of our serological results, PPV-D and PPV-M could be regarded as different viruses. This conclusion is, however, not supported by molecular data: on the basis of molecular parameters such as coat protein nucleic acid sequences (SHUKLA and WARD 1989a,b, WARD and SHUKLA 1991), and nucleotide sequence of the 3′-untranslated region (FRENKEL *et al.* 1989, FRENKEL *et al.* 1992), all PPV isolates

Table 2

Correlation between electrophoretic mobilty of coat protein subunits, immunological properties of N- and C-terminal domains of coat protein, and RsaI restriction site polymorphism in 3′ end of PCR amplified coat protein gene

	Coat protein			PCR Amplified Coat protein gene	
	Electrophoretic mobility		IgG anti PPV-D[1] to N- and C-terminal parts	*Rsa* I[2] Restriction site	
Isolate	Fast	Slow			Group
F117	F		+	+	D
Dideron	F		+	+	D
F97		S	0	0	M
F116	F		+	+	D
F118		s	0	0	M
F63	F		+	+	D
F96		s	0	0	M
F119	F		+	+	D
F14		s	0	0	M
F66	F		+	+	D
F67	F		+	+	D
F24		s	0	0	M
F120		s	0	0	M
Y2744		s	0	0	M
R4776	F		+	+	D
G1	F		+	+	D
A58	F		+	+	D
A123	F		+	+	D
Y100		s	0	0	M
El-Amar		s	0	0	M
Markus		s	0	0	M
B71		s	0	0	M
G98	F		+	+	D
S72	F		+	+	D
Y101		s	0	0	M
Y56	F		+	+	D
T60		s	0	0	M
R59	F		+	+	D

[1] reaction with antibodies, 0: no reaction.
[2] presence of restriction site, 0: no restriction site.

sequenced so far correspond to a single virus (WETZEL *et al.* 1991b; CERVERA *et al.* 1993). By this criterion, three distinct PPV strains can be proposed: PPV-D, PPV-NAT and PPV-R would be isolates of the same strain (SHUKLA *et al.* 1989), PPV-El-Amar would be a second distinct strain (WETZEL *et al.* 1991b) and PPV-PS would represent a third strain (CERVERA *et al.* 1993).

There are significant divergencies in the N-terminal part of the coat protein gene of the sequenced isolates: 54.3% in predicted amino acid sequence between PPV-El-Amar with group D consensus (PPV-D, PPV-NAT, and PPV-R), 43.6% between PPV-El-Amar and PPV-PS, and 26.1% between group D consensus and PPV-PS (WETZEL *et al.* 1991b, CERVERA *et al.* 1993). These observations suggest that our N- and C-terminal serological

results, obtained using only antibodies to one serotype (D), reflect only part of the existing serodiversity. Indeed, serogroup M as defined in this study can be regarded as a collection of isolates that do not react in the same way as those of group D. Especially in the case of the N- and C-terminal regions serological analysis, group M isolates are defined as those showing no cross reactions with PPV-D. These results, therefore, do not exclude the existence of greater serological diversity within the serogroup M.

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