# DETECTION AND BIODIVERSITY OF CUCUMBER MOSAIC CUCUMOVIRUS. CONCLUSIONS FROM A RINGTEST OF EUROPEAN UNION COST 823 (NEW TECHNOLOGIES TO IMPROVE PHYTODIAGNOSIS)

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## SUMMARY

The detectability and biodiversity of 44 isolates of cucumber mosaic virus (CMV), one isolate of peanut stunt virus (PSV) and one of tomato aspermy virus (TAV), all cucumoviruses, were compared by RT-PCR, variants of ELISA using polyclonal and monoclonal antibodies (Pabs and Mabs) and host plant reactions. The comparisons were made during a Ringtest on zucchini viruses held at the Istituto di Fitovirologia Applicata, CNR, Torino, Italy, in October 1996 in the framework of the European Union action COST-823 «New technologies to improve phytodiagnosis».

The isolates originated from 9 European countries, America, Asia and Australia and their natural hosts were mainly cucurbits. Some of the isolates had been characterized previously.

RT-PCR with primers specific for CMV RNA 3 followed by digestion with *Msp*I allowed the classification of 19 isolates within subgroup I (S-I) and 13 in subgroup II (S-II). Three distinct and different S-I-like patterns were found for 8 isolates, while 3 isolates displayed a new pattern called S nonI-nonII. PSV, TAV and one isolate were not amplified with this primer set. Three RNA 2-based sets of primers, specific for S-I, S-II or both, substantiated the above results.

In DAS ELISA Pabs produced against S-I detected most of CMV isolates, but not PSV and TAV, whereas Pabs produced against S-II isolates detected mainly those. In ACP ELISA, two Pabs detected all the cucumoviruses tested. In TAS ELISA, 7 Mabs reacted with most of the isolates and none of them reacted with PSV and TAV. Two Mabs were specific for S-I and two for S-II, confirming the PCR results.

The isolates induced symptoms of different severity after inoculation to zucchini, *Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. tabacum* and *Chenopodium quinoa* but no correlation was found with subgroup type. Seven S-I isolates and one S-II isolate gave systemic infection on *C. quinoa*.


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All the isolates were deposited at the *Deutsche Sammlung von Mikroorganismen und Zellkulturen*, Germany, and are available for research purposes.


## RIASSUNTO

**Rilevamento e biodiversità di cucumber mosaic cucumovirus. Conclusioni da un "Ringtest" dell'Unione Europea cost 823 (Nuove Tecnologie per migliorare la fitodiagnosi)**. La tecnica della RT-PCR, varianti ELISA che prevedevano l'utilizzo di anticorpi monoclonali e policlonali, e la reazione su piante ospiti, sono state utilizzate per comparare la rilevabilità e la biodiversità di 44 isolati di cucumber mosaic (CMV), uno di peanut stunt (PSV) e uno di tomato aspermy (TAV) cucumovirus. La comparazione è stata effettuata nel corso di un Ringtest denominato "Zucchini Viruses", effettuato nell'ottobre 1996 presso l'Istituto di Fitovirologia Applicata e organizzato dall'azione COST 823 "Nuove tecnologie per migliorare la fitodiagnosi" dell'Unione Europea.

Gli isolati provenivano da 9 nazioni europee, America, Asia e Australia e le piante ospiti originali erano prevalentemente cucurbitacee. Alcuni isolati erano stati caratterizzati precedentemente.

RT-PCR effettuata con primers specifici per l'RNA 3 di CMV e digestione del segmento amplificato con *Msp*I ha permesso di classificare 19 isolati come sottogruppo I (S-I) e 13 come sottogruppo II (S-II); otto isolati hanno mostrato un quadro elettroforetico diverso ma riconducibile a quello del S-I, mentre 3 isolati hanno mostrato un quadro elettroforetico completamente diverso, denominato nonI-nonII. Gli isolati di PSV e TAV e un ulteriore isolato non sono stati amplificati correttamente con questa coppia di primers. Tre coppie di primers basati sulla sequenza dell'RNA 2 di CMV e specifici per il S-I, S-II o entrambi hanno fornito risultati in accordo con i precedenti.

In DAS ELISA gli antisieri prodotti contro isolati del S-I hanno rilevato la maggior parte degli isolati di CMV ma non PSV e TAV, mentre quelli prodotti contro isolati del S-II hanno rilevato prevalentemente i

virus membri dello stesso sottogruppo. In ACP-ELISA due antisieri policlonali prodotti contro CMV hanno rilevato tutti i cucumovirus saggiati. In TAS ELISA nessuno degli anticorpi monoclonali prodotto contro CMV ha reagito con PSV e TAV; 7 di loro hanno reagito con la maggior parte degli isolati mentre 2 sono risultati specifici per gli isolati del S-I e 2 per gli isolati del S-II, confermando i risultati ottenuti con la PCR.

Gli isolati hanno indotto sintomi di diversa gravità in zucchino*, Nicotiana benthamiana, N. glutinosa, N. tabacum e Chenopodium quinoa* e non hanno mostrato alcuna correlazione con la loro suddivisione nei due sottogruppi. Sette isolati del S-I e uno del S-II hanno prodotto infezione sistemica in *C. quinoa.*

Tutti gli isolati sono stati depositati presso la *Deutsche Sammlung von Mikroorganismen und Zellkulturen*, Germany, e sono disponibili per uso ricerca.

*Key words:* plant virus, diagnosis, cucumovirus, RT-PCR, ELISA, zucchini.

## INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* (Bromoviridae) which also includes peanut stunt virus (PSV) and tomato aspermy virus (TAV) (Murphy *et al.*, 1995). CMV is considered one of the five most important viruses infecting horticultural crops (Tomlinson, 1987), because it infects over 800 plant species, is present world-wide and is transmitted by many aphid species in the non-persistent manner. Due to its economic importance the detection and differentiation of CMV isolates using different techniques has been reported in many papers.

The genome of CMV consists of three single-stranded positive-sense RNAs, each encapsidated in a separate particle approximately 30 nm in diameter. RNA 1 and 2 encode proteins involved in virus replication; RNA 3 directly encodes the movement protein, and indirectly the coat protein via a subgenomic RNA. The coat protein consists of 218-220 amino acids corresponding to a mol. wt of about 24.5 kDa. Some isolates also carry a satellite RNA of 330-405 nucleotides, affecting symptom expression and virus replication mainly in solanaceous hosts (Palukaitis *et al.*, 1992).

According to biological, serological and nucleic acid properties, CMV isolates are placed in two main subgroups, historically given different names as summarized by Palukaitis *et al.* (1992). These subgroups are now commonly referred as Subgroup I (S-I) and Subgroup II (S-II). Apparently there are no differences in the host range of S-I and S-II isolates. Studies on RNA, based on sequencing and hybridization tests, confirm the subdivision into S-I and S-II. Whichever RNA segment is considered, nucleotide sequence homologies between S-I and S-II range from 71 to 76%, while within each subgroup they are above 92%. Serologically the two subgroups are closely related as shown by cross reactions of polyclonal antibodies leading to serological differentiation index (SDI) values of 1-2 among S-I and S-II isolate, whereas the SDI separating PSV, TAV and either of the CMV subgroups is 6-7 (Palukaitis *et al.*, 1992). Monoclonal antibodies (Mabs) produced against S-I and II isolates have differing specificities but few of them can differentiate the two subgroups, indicating the presence of unique epitopes for each (Haase *et al.*, 1989; Porta *et al.*, 1989).

Since 1988 the European Union has funded the COST Action 88 'Early Detection of Plant Pathogens' programme, which was followed in 1995 by the COST action 823 'New technologies to improve phytodiagnosis'. The aim was to improve the diagnosis of plant pathogens by means of exchange of information, creation of databases and establishment of permanent reference collections of pathogens. Among the different activities, the so-called 'Ringtest' has proved valuable for a better harmonization of phytodiagnosis in Europe. A Ringtest involves comparison of a large number of virus isolates, antibodies and nucleic acid probes under identical conditions at the same time, preferably done by all the people directly involved, to establish ranges of variability of the isolates, and the reliability of detection and differentiation of the viruses with the different reagents. So far, four Ringtests have been held relating to potato Y potyvirus, (1991) in Braunschweig, Germany; plum pox potyvirus, (1993), in Valencia, Spain; tospoviruses, (1995) in Wageningen, The Netherlands (Adam *et al.*, 1996); and Zucchini viruses, (1996) in Torino, Italy (see at http://www.ifgb.ifgb.uni-hannover.de/extern/ppigb/COST823/index.htm). The last Ringtest focussed on a crop rather than a single virus, due to the economic importance of a restricted number of viruses in the zucchini crop (Lovisolo, 1981; Blancard *et al.*, 1991; Zitter *et al.*, 1996). Here we report the results pertaining to CMV whereas the results for the potyviruses will be reported elsewhere. To profit from the concentration of expertise and technology afforded by the Ringtest, the CMV isolates compared came from other crops as well as zucchini, and not only from Europe but worldwide.

## MATERIALS AND METHODS

**Ringtest**. The Ringtest was hosted at the Istituto di Fitovirologia Applicata (IFA), CNR, Torino, Italy, in October, 1996. To avoid errors in the handling of data, the original Ringtest numbers of the virus isolates as

**Table 1.** Participants and suppliers of viruses, antisera and primers for the Ringtest.

| | |
|---|---|
| G.P. ACCOTTO | Institute of Applied Plant Virology, CNR, Torino, Italy |
| G. ADAM | Dept. of Plant Protection, Institute of Applied Botany, University of Hamburg, Germany |
| D. ALIOTO | Institute of Plant Pathology, Faculty of Agronomy, Portici, Napoli, Italy |
| A. BASALP | Marmara Research Centre, Gebze-Kocaeli, Turkey |
| K. BECH | Dept. of Plant Pathology and Pest Management, Danish Institute of Agric. Sci., Lyngby, Denmark. |
| C. CUPERUS | Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands |
| G. DELLAVALLE | Institute of Applied Plant Virology, CNR, Torino, Italy |
| C. DESBIEZ. | Plant Pathology Station, INRA, Montfavet, France |
| G. GRASSI | Institute of Industrial Crops, MIPA, Bologna, Italy |
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| G. NOLASCO | UCTA, Campus de Gambelas, University of Algarve, Faro, Portugal |
| F. RABENSTEIN | Institute for Pathogen Diagnostics, FCBRCP, Aschersleben, Germany |
| P. ROGGERO | Institute of Applied Plant Virology, CNR, Torino, Italy |
| L. TOMASSOLI | Institute of Plant Pathology, MIPA, Roma, Italy |
| L. TORRANCE | Scottish Crop Research Institute, Dundee, Scotland |
| R. van der VLUGT | Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands |
| A.M. VAIRA | Institute of Applied Plant Virology, CNR, Torino, Italy |
| J. VETTEN | Institute of Biochemistry and Plant Virology, BBA, Braunschweig, Germany |
| S. WINTER | Plant Virus Collection, DSMZ, Braunschweig, Germany |

well as the number of the ELISA plate, indicating the different combinations of antibodies used, will be maintained throughout the text. Participants and suppliers of virus isolates and antisera are listed in Table 1. For sequence information on cucumoviruses see http:// biology.anu.edu.au/Groups/MES/vide/genus0.14.htm (Brunt *et al*., 1996).

**Virus isolates and host plants.** The isolates used are listed in Table 2, together with other relevant information. They were sent to IFA over a period of 4 months by the participants and donors as freeze-dried or fresh leaves. The samples were sap inoculated using standard procedures to zucchini and *Nicotiana benthamiana* and maintained on these plants. Two weeks before the Ringtest, all isolates were inoculated at the same time to at least 4 plants of each of the following species: *Chenopodium quinoa, Cucurbita pepo* cv. 'Genovese', *N. benthamiana, N. glutinosa* and *N. tabacum* White Burley. All plants were maintained under strict containment in a glasshouse at around 25/18°C day/night temperature. Symptoms of infection were visually evaluated by a panel of three participants, and severity of infection was scored from 0 to 3, 15 days after inoculation.

At the end of the Ringtest all the isolates were freeze-dried and, with permission from the donors, they were sent to the *Deutsche Sammlung von Mikroorganismen und Zellkulturen,* (DSMZ), Braunschweig, Germany for permanent storage in a separate collection for research use only.

**Nucleic acid analysis**

*RNA extraction.* Total plant RNA was extracted using the UltraSpec RNA extraction reagent (Biotecx Laboratories, Inc., Houston, Texas, USA) according to manufacturer's instructions. Approximately 0.2 g of

young zucchini leaf with symptoms (or *N. benthamiana* for isolates not infecting zucchini systemically) were frozen in liquid nitrogen and homogenised with 1 ml of the extraction solution. The RNA pellets obtained by isopropanol precipitation and centrifugation were resuspended in 40 µl of DEPC-treated water.

*PCR primers.* The primers used are listed in Table 3. Primers 5'CP (-) and 3'CP (+) (Rizos *et al.*, 1992) are based on CMV-Q RNA 3 and amplify an 870 bp fragment for all CMV isolates, which can be cut by the restriction enzyme *Msp*I to obtain patterns distinguishing S-I from S-II isolates. Primers LS 2.2 (-) and LS 0.8 (+) based on the sequence of CMV-LS RNA2 (Marilyn Roossinck, Noble Foundation, Ardmore, USA, personal communication), amplify a 1400 bp fragment in S-II isolates. Primers LS2.2 (-) and FNY1.8 (+) (based on CMV-FNY RNA 2) amplify a fragment of about 400 bp in S-I isolates. Primers CMV1 (-) and CMV2 (+) based on the RNA 2 sequence of isolate FNY (Karl-Heinz Hellwald, Peter Palukaitis Scottish Crop Res. Inst. Dundee, Scotland, UK, personal communication), amplify a 400 bp fragment in both S-I and S-II isolates.

*Reverse transcription.* Total RNA (1 µl) was denatured at 65°C for 2 min and added to 9 µl of the RT reaction mix (containing 4 mM NaPP, 2 units RNase inhibitor, 1 mM of each dNTPs, 10 units AMV reverse transcriptase [Invitrogen BV, Leek, The Netherlands] and 10 ng of the negative polarity primer). The preparation was incubated at 42°C for 45 min and then diluted 1/5 with water.

*PCR.* Amplification conditions are given in Table 4. The PCR reaction volume was 25 µl; the reaction mix, containing 200 µM each of the four dNTPs, 0.2 µM of each primer, 2 mM $MgCl_2$, 0.6 units of AmpliTaq (Perkin Elmer) and 1 µl of diluted cDNA as template, was subjected to thermal cycling using the Perkin Elmer GeneAmp PCR System 2400. Following PCR, 10 µl of product was electrophoresed in a 1% agarose gel, then stained with ethidium bromide. After *Msp*I digestion, the DNA was electrophoresed in 3% NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME, USA) and stained as above.

**Serology**

*Antibodies.* The polyclonal and monoclonal antibodies (Pabs and Mabs) used are listed in Tables 5a, b and c, along with the plate number and any additional information available. The antibodies were either supplied as crude serum, culture supernatant or in the form of purified IgG and alkaline phosphatase-conjugated IgG for the Pabs.

*ELISA protocols.* To obtain the maximum of information about the antibodies and their reaction with the virus isolates, three different ELISA procedures were employed: antigen-coated plate (ACP), double antibody sandwich (DAS) and triple antibody sandwich (TAS), using standard buffers (Clark and Adams, 1977). The plates were of the high protein binding type (Greiner, Kremsmünster, Austria). The dispensed volume was 100 µl per well at each step and the plates were shaken using an ELISA plates shaker (Ika, Staufen, Germany). They were washed three times between each step using a plate washer (BIO RAD, Hercules, CA, USA, Mod. 1550 ) set to a volume of 200 µl per well. Final absorbance was read at 405 nm and 490 nm using a BIO RAD Automatic Reader 3550. The results were transferred to a spreadsheet programme for further analysis. For each combination of antibodies three plates were used, therefore each number represents three plates.

In each plate the following controls were included: 2 wells filled with water as blank, 2 wells filled with all the reagents and PBS-Tween as antigen, 8 wells filled with sap from healthy plants diluted 1/50 and 1/500 (w/v) and 4 wells filled with sap from plants infected by the homologous virus, when available, at the same two dilutions. Each sample was tested at dilutions of 1/50 and 1/500 (w/v), each replicated in 2 wells.

For DAS- and TAS-ELISA, plates were coated with the antibodies diluted in 0.1 M carbonate buffer, pH 9.6, and incubated at room temperature for at least 6 h.

Samples consisted of systemically infected zucchini leaves. For the few isolates not infecting zucchini, leaves of *N. benthamiana* were used. The samples were homogenised 1/10 (w/v) in plastic bags (Bioreba, Reinach, Switzerland) with 10 mM phosphate buffer pH 7, plus 20 mM $Na_2SO_3$ and 10 mM Na-DIECA. The extracts were immediately diluted with carbonate buffer for ACP-ELISA or with PBS Tween plus 2% polyvinylpyrrolidone (PVP) MW 30,000, for DAS- and TAS-ELISA. The plates were incubated overnight at 4°C.

Alkaline phosphatase conjugates or unlabelled antibodies were diluted with PBS Tween plus 2% PVP. For ACP-ELISA the rabbit Pabs were incubated for 4 h at room temperature and bound antibodies were detected using goat anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, Missouri, USA) incubated for 1 h at room temperature. In DAS-ELISA the alkaline phosphatase conjugates were incubated for 4 h, and for TAS ELISA the Mabs were incubated for 4 h, followed by a rabbit anti-mouse alkaline phosphatase conjugate (Sigma), for 1 h. The substrate was 0.8 ng $ml^{-1}$ of p-nitrophenylphosphate in diethanolamine buffer, pH 9.8. Plates were incubated at room temperature and absorbance was read after about 90 min.

For evaluating the plates the corresponding blank values were subtracted for each plate separately. Thereafter the values of the three plates for each combination of antibodies were combined and analysed according to Rek (1987) to determine the cut-off values for positive samples. Values below the cut-off limit were indicated as negative (-) and the remaining positive values were converted into a plus (+) scheme with values ranging from + to ++++, with actual absorbance values ranging from 0.1 to 2.7. Evaluation of the intensity of reactions was done grouping all the plates of the same ELISA format. This evaluation allowed comparison of data from three plates, giving results similar to the more common method of evaluating positive/negative results based on absorbance ratio between test and healthy samples, and taking 3 as positive values (data not shown).

The numbers used below to identify isolates are the ringtest (RT) code numbers given in Table 2.

## RESULTS

**Virus isolates**. A total of 44 isolates of CMV were analyzed during the Ringtest; one isolate of PSV (104) and one of TAV (100) were included for comparison. Of the CMV isolates, 23 originally came from cucurbits. The isolates came from 17 countries, 9 European, 5 Asian, 2 American and one Australian. The date of the original isolation ranged from 1957 to 1996, with 1995 and 1996 isolates being the most frequent (Table 2).

**PCR amplification and analysis**. Extraction of RNA using the procedure described was successful and rapid enough to allow processing of all samples in a short time. All isolates were tested with primer pair 5'CP/3'CP and some of them, selected randomly before the beginning of the experiments, were also tested with other primer pairs. All the data are summarised in Tables 6 and 7.

With primers 5'CP/3'CP a fragment of about 870 bp was amplified for all samples except 104 (PSV), 100 (TAV) and 140 which each showed differing complex amplification patterns (Fig. 1). A second smaller less intense band was found for most isolates, more easily visible when the first band was less intense.

After digestion of the amplified product with *Msp*I and electrophoresis (Fig. 2, Tables 6 and 7) 19 isolates gave two bands of 540 and 340 bp and were classified as S-I; 13 isolates gave 4 bands of 250, 200, 160 and 130 bp and were classified as S-II, according to Rizos *et al.* (1992). Results obtained with the other isolates were different: for 6 isolates identified as S-Iα the pattern was very similar to S-I but the 340 bp fragment was substituted by a smaller one. For one isolate, identified as S-Iβ, the pattern was again similar to S-I but the 540 bp fragment was substituted by a larger one. Isolate 144 designed S-Iγ gave 3 bands, the middle comigrating with the middle band of isolate S-Iα. Three isolates (6, 48 and 85) showed a pattern of 4 bands, none of which corresponded with those of S-I or S-II isolates; for this reason they were identified as 'nonI-nonII'.

With primer pairs located on CMV RNA 2 good correlation was found with the results obtained with 5'CP/3'CP. However with primers CMV1/CMV2, amplifying both S-I and S-II, only 5 of the 6 isolates classified as S-I resulted in an amplified product and only 7 of the 8 isolates belonging to S-II were amplified. When 4 of the isolates classified as S-Iα and one classified as 'nonI-nonII' were tested, a product was amplified. This primer pair also yielded amplified fragments with PSV and TAV. With primers LS2.2/LS0.8, specific for S-II, the isolates classified as S-I, S-Iα and 'nonI-nonII' were negative whereas the five isolates classified as S-II were positive.

To simplify grouping of the isolates and to compare with the results of serology and host plant assays, the classification obtained with primers 5'CP/3'CP will be used in the text.

**Serology**. Extracts tested at dilutions 1/50 and 1/500 gave similar results in all ELISA formats, indicating a high concentration of viral antigen in all samples. Absorbances corresponding to the 1/50 dilution were used for handling of data. Results are given in detail in Table 8 and summarized in Table 9.

**DAS-ELISA**. The 6 combinations tested gave different results. Plates 1, 4 and 10 with antisera against S-II isolates, showed different degrees of specificity, with plate 1 detecting only one, but plate 10 detecting 10 out of 19 S-I isolates. Plates 7, 13 and 16, with antisera against S-I isolates, were apparently less specific, detecting most CMV isolates, although with stronger reactions for S-I isolates; 43 and 41 isolates were detected respectively on plates 7 and 13. No reaction was found with TAV (100), PSV (104) and isolate 140. Isolates 139 and 144 were detected with only two antisera.

**ACP-ELISA**. All isolates including PSV and TAV were detected on plates 52 and 55, except isolate 139 on plate 55. In plate 61, the same antiserum as in DAS-ELISA plate 4 was used; however in the ACP format 40 isolates were detected instead of 18. No reaction was found with PSV, TAV and isolates 139, 140 and 144.

**Table 2.** Cucumovirus isolates tested during the Ringtest.

| Donor | RT code | Natural host | Original label | Year of isolation | Origin | Country | References |
|---|---|---|---|---|---|---|---|
| Katis | 3 | Cucumber | 3 | 1995 | Korinthos | GR | |
| | 6 | Melon | 6 | 1995 | Akrefnio, Voiotia | GR | |
| | 8 | Melon | 8 | 1995 | Preveza | GR | |
| | 10 | Watermelon | 10 | 1996 | Livanates | GR | |
| Miller | 21 | Zucchini | Carmello | 1993 | Evesham | UK | |
| Lecoq | 38 | Melon | 14 | 1977 | Avignon | FR | Lecoq *et al.*, 1979 |
| | 39 | Squash | Colmar | 1992 | Colmar | FR | |
| Tomassoli | 48 | Squash | 643/96 | 1996 | Lazio | IT | |
| | 51 | Melon | 57/92 | 1992 | Lazio | IT | |
| Hellwald | 52 | Squash | Fny | na | New York | US | Palukaitis *et al.*, 1992 |
| | 54 | Cornflower | K | 1982 | China | CN | Tien-Po *et al.*, 1982; Hellwald and Palukaitis, 1994. |
| | 55 | Tomato | PRC-CMV | 1989 | China | CN | Kearney *et al.*, 1990 |
| Van der Vlugt | 67 | Lettuce | Ls083 | 1978 | Groessen | NL | |
| | 68 | Spinach | Sp U1 | 1974 | Duiven | NL | |
| | 69 | Spinach | Sp43 | 1982 | Lelystad | NL | |
| | 70 | Pea | Uk6 | 1957 | na | na | |
| | 71 | na | K8 | na | na | na | |
| | 72 | na | S4 | na | na | na | |
| Luis-Arteaga | 85 | Melon | B20.2 | 1995 | Barcelona | ES | |
| | 86 | Melon | M/95/33 | 1995 | Badajoz | ES | |
| | 87 | Melon | M6 | 1995 | Valencia | ES | |
| | 88 | Melon | M-12-96 | 1996 | Navarra | ES | |
| | 89 | Melon | M-20-96 | 1996 | Zaragoza | ES | |
| | 90 | Melon | M-3-96 | 1996 | Zaragoza | ES | |
| Bech | 96 | *Beta vulgaris* | 2:7:2 | 1972 | From M.Hollings | UK | |
| | 97 | Cucumber | 2:10:2 (CMV-14) | na | From H. Lecoq | FR | |
| Winter | 99 | Cucumber | PV0187 | na | Deggendorf | GE | |
| | 100 | Tomato | TAV-PV-0220 | na | na | BG | |
| | 101 | *Vinca major* | CMV II | 1992 | California | US | Daniels and Campbell, 1992 |
| | 102 | Pepper | CMV Ia | 1992 | California | US | Daniels and Campbell, 1992 |
| | 103 | Pepper | CMV Ib | 1992 | California | US | Daniels and Campbell, 1992 |
| | 104 | na | PSV-PV-0190 | na | na | US | |
| | 107 | Zucchini | PV-0453 | na | Taiwan | TW | |
| Nolasco | 109 | Melon | TAV | 1995 | Algarve | PT | |
| | 110 | Melon | FAR | 1995 | Algarve | PT | |
| Lisa | 135 | Bean | F 100 | 1976 | Piemonte | IT | |
| | 136 | Carnation | car | 1967 | Liguria | IT | Lovisolo *et al.*, 1968 |
| | 137 | Zucchini | Z 160 | 1990 | Piemonte | IT | |
| | 138 | Bean | VE 185 | 1989 | Shiraz | IR | |
| | 139 | Bean | VE 230 | 1991 | Chile | CL | |
| | 140 | Bean | Tu 56 | 1988 | Erzurum | TR | |
| | 141 | Bean | Tu 86 | 1988 | Erzurum | TR | |
| | 142 | Bean | Ch 93 | 1990 | Yianquin | CN | |
| | 144 | Bean | VE 97 | 1988 | Plovdiv | BG | |
| | 145 | Zucchini | VE 269 | 1996 | Amman | JO | |
| Torrance | 146 | Lupin | LW | na | From R.A.C. Jones | AU | |

na: not available

**Table 3.** Primers used for PCR amplification.

| Primer | Polarity | Sequence | Target | Reference |
|---|---|---|---|---|
| 5'CP | - | 5'-CTCGAATTCGGATCCGCTTCTCCGCGAG-3' | RNA 3 | Rizos *et al.*, 1992 |
| 3'CP | + | 5'-GGCGAATTCGAGCTCGCCGTAAGCTGGATGGAC-3' | | |
| CMV-1 | - | 5'-GATCATCGCCTGAGAATA-3' | RNA 2 | Hellwald and Palukaitis, 1994 and personal communication |
| CMV-2 | + | 5'-TTCCAGAGATGCCTTCG-3' | | |
| LS 2.2 | - | 5'-AGTTAGCGGTGTACTTCTTA-3' | RNA 2 | Rezaian *et al.,* 1984; M. Roossinck, personal communication |
| LS 0.8 | + | 5'-TTCTTATTCAAAGACCGAGG-3' | | |
| FNY 1.8 | + | 5'-CACCGACCAATTCGAAAAGC-3' | RNA 2 | Hellwald, personal communication |

**Table 4.** PCR conditions employed for amplification after reverse transcription.

| Primer pair | Cycles | Denaturation | Annealing | Extension | Product (bp) | Specificity (Subgroup) |
|---|---|---|---|---|---|---|
| 5'CP /3'CP | 40 | 94°C, 30 sec | 50°C, 30 sec | 72°C, 30 sec | 870 | I or II after *Msp*I digestion |
| CMV-1 /CMV-2 | 35 | 94°C, 30 sec | 50°C, 30 sec | 72°C, 60 sec | 400 | I and II |
| LS 2.2 / LS 0.8 | 35 | 94°C, 30 sec | 50°C, 30 sec | 72°C, 90 sec | 1400 | II |
| LS 2.2 / FNY 1.8 | 30 | 94°C, 60 sec | 55°C, 60 sec | 72°C, 120 sec | 400 | I |

**TAS-ELISA**. High variability and specificity was observed with all the combinations. Specificity depended on the Mabs employed because only 2 different Pabs were used for coating (one for 19, 22, 25 28 and 31 and another for 34, 37, 40, 43 46 and 49) and the reactions were specific for either S-I or S-II or detected both, independently of the coating antibodies. The Mabs could be placed in at least 3 groups: A, reacting with most or all isolates (19, 22, 28, 31, 34, 43 and 49); B, generally reacting with S-I only (25 and 40); C, reacting with S-II only (37 and 46). Isolate 139 was detected only on plate 22, isolate 144 on 6 plates, and isolate 140, PSV and TAV were not detected on any plate.

**Isolates with low detectability.** Considering the overall ELISA results (see Table 8, last column), 4 isolates were detected in a low number of plates: isolate 103 (S-I) in 9 combinations, isolate 139 (S-II) in 4 combinations, isolate 140 in ACP-ELISA with the antibodies also reacting with PSV and TAV, and isolate 144 detected in 10 combinations.

**Host range.** All isolates induced symptoms about 6 days after inoculation. Detailed results are given in Table 10 and summarized in Table 11.

Only isolates 140 and 146 as well as PSV and TAV did not infect zucchini. For most others, symptoms were severe and consisted of chlorosis, mosaic and stunting. Most isolates infected the solanaceous test plants systemically with different symptom intensity. All isolates induced local lesions in *C. quinoa* except isolate 51. Eight isolates, including 51, infected *C. quinoa* systemically and 7 of these belonged to S-I.

## DISCUSSION

As far as we know this is one of the largest simultaneous comparisons of detection and differentiation methods applied to CMV isolates coming from many parts of the world. Inclusion in the Ringtest of well known isolates such as Fny and others provides the basis for a comparison with previous work. All the isolates used are now deposited at the DSMZ as a permanent collection, available for research.

**Table 5a.** Antisera used in DAS ELISA.

| Plate No. | Donor | Original label of antiserum | Homologous virus (RT code) |
|---|---|---|---|
| 1 | Lisa | A326 | 136 |
| 4 | Lisa | A340 | 136 |
| 7 | van der Vlugt | IPO/S4 | 72 |
| 10 | van der Vlugt | IPO/K8 | 71 |
| 13 | Lecoq | na | 38 |
| 16 | Winter | 803 | 99 |

**Table 5b.** Antisera used in ACP ELISA.

| Plate No. | Donor | Original label of antiserum | Homologous virus (RT code) |
|---|---|---|---|
| 52 | Torrance | na | na |
| 55 | Nolasco | EAN-1991 | na |
| 61 | Lisa | A340 | 136 |

**Table 5c.** Antibodies used in TAS ELISA.

| Plate No. | Original label of polyclonal rabbit antibodies for coating plates | Donor | Homologous virus (RT code) | Original label of monoclonal antibodies | Homologous virus (RT code) | Donor |
|---|---|---|---|---|---|---|
| 19 | A24 | Luisoni | 136 | M2 | 135 | Grassi |
| 22 | | | | M85 | | |
| 25 | | | | M172 | | |
| 28 | | | | M176 | | |
| 31 | | | | M185 | | |
| 34 | na | Vetten | na | CF11 | na | Rabenstein |
| 37 | | | | BD9 | | |
| 40 | | | | 1-5G7 | na | Vetten |
| 43 | | | | 2-4E3 | | |
| 46 | | | | LB-3G6 | | |
| 49 | | | | I-5G9 | | |

**Table 6.** Detection and differentiation of cucumovirus isolates by RT-PCR.

| Virus isolates (RT no.)[a] | Primer pairs | | | | |
|---|---|---|---|---|---|
| | 5'CP / 3'CP | | CMV1/CMV2 (Subgroups I and II) | LS2.2/FNY1.8 (Subgroup I) | LS2.2/LS0.8 (Subgroup II) |
| | 870 bp fragment | Subgroup assignment after *Msp*I digestion | 400 bp fragment | 400 bp fragment | 1400 bp fragment |
| 3 | + | I | | | |
| 8 | + | I | | | |
| 10 | + | I | | | |
| 51 | + | I | + | + | – |
| 52 | + | I | + | + | – |
| 72 | + | I | | | |
| 86 | + | I | | | |
| 87 | + | I | | | |
| 88 | + | I | | | |
| 89 | + | I | + | + | – |
| 90 | + | I | | | |
| 99 | + | I | | | |
| 103 | + | I | + | + | – |
| 109 | + | I | + | | |
| 110 | + | I | | | |
| 135 | + | I | – | | |
| 137 | + | I | | | |
| 141 | + | I | | | |
| 145 | + | I | | | |
| 38 | + | Iα | + | + | – |
| 54 | + | Iα | + | | |
| 55 | + | Iα | | | |
| 97 | + | Iα | | | |
| 107 | + | Iα | + | | |
| 142 | + | Iα | + | | |
| 138 | + | Iβ | | | |
| 144 | + | Iγ | | | |
| 21 | + | II | + | – | + |
| 39 | + | II | + | – | + |
| 67 | + | II | + | – | + |
| 68 | + | II | | | |
| 69 | + | II | | | |
| 70 | + | II | + | – | + |
| 71 | + | II | | | |
| 96 | + | II | | | |
| 101 | + | II | + | | |
| 102 | + | II | + | – | + |
| 136 | + | II | – | | |
| 139 | + | II | + | | |
| 146 | + | II | | | |
| 6 | + | nonI-nonII | + | + | – |
| 48 | + | nonI-nonII | | | |
| 85 | + | nonI-nonII | | | |
| 140 | - | | | | |
| 100 | - | | + | | |
| 104 | - | | + | | |

[a] Isolates sorted according to results with primers 5'CP/3'CP and *Msp*I restriction. Blanks indicate not tested.

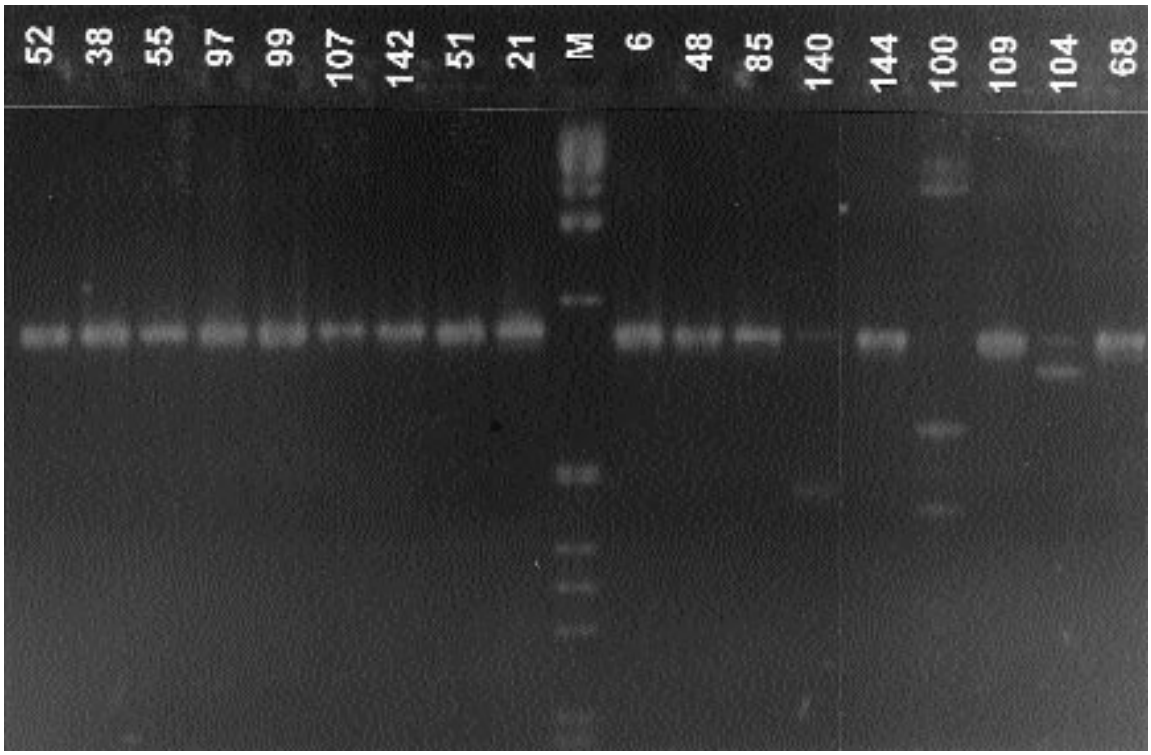
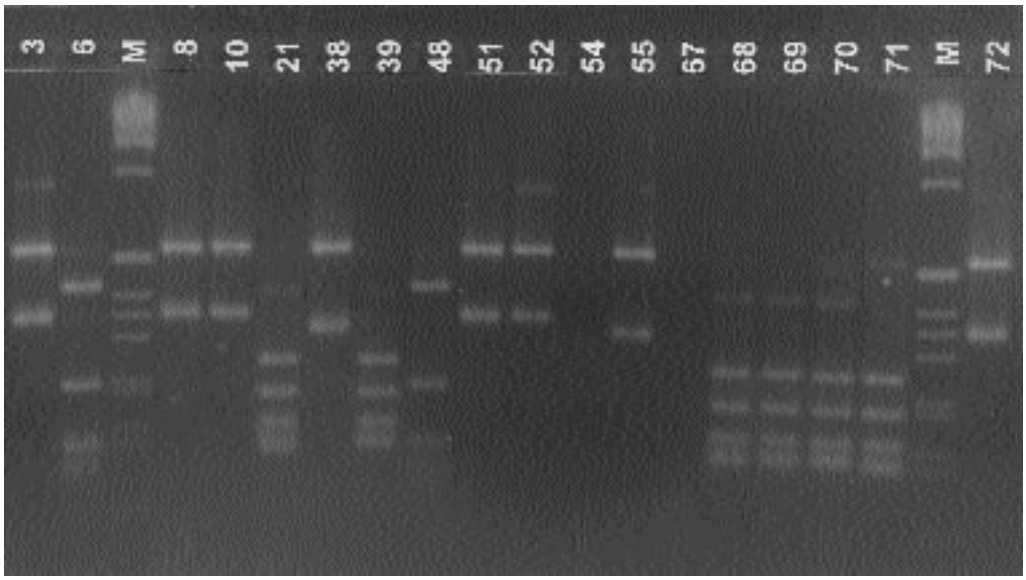



**Fig. 1.** Electrophoretic analysis in 1% agarose of the amplified products obtained for some cucumovirus isolates with primers 5'CP /3'CP. Gels stained with ethidium bromide. M indicates molecular weight markers. The major band corresponds to 870 bp.

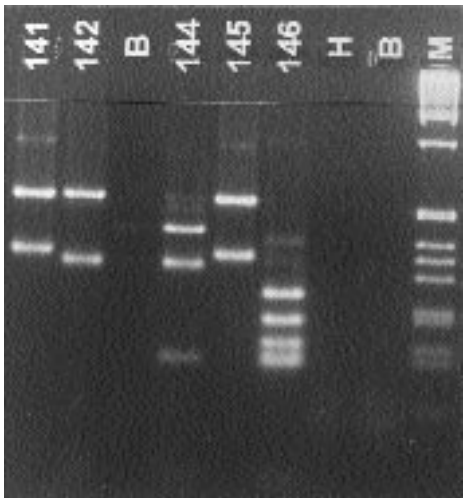
**Table 7.** Summary of the detection of cucumoviruses by RT-PCR. Results are expressed as positive samples over tested.

| Subgroup [a] | Primer pairs | | | |
|---|---|---|---|---|
| | 5'CP/3'CP plus *Msp*I digestion | CMV1/CMV2 (Subgroups I and II) | LS2.2/FNY1.8 (Subgroup I) | LS2.2/LS0.8 (Subgroup II) |
| S-I | 19 | 5/6 | 4/4 | 0/4 |
| S-Iα | 6 | 4/4 | 1/1 | 0/1 |
| S-Iβ | 1 | nt | nt | nt |
| S-Iγ | 1 | nt | nt | nt |
| All S-I like | 27 | 9/10 | 5/5 | 0/5 |
| S-II | 13 | 7/8 | 0/5 | 5/5 |
| nonI-nonII | 3 | 1/1 | 1/1 | 0/1 |
| 140 | – | nt | nt | nt |
| TAV | – | 1/1 | nt | nt |
| PSV | – | 1/1 | nt | nt |
| Total | 43/46 | 19/21 | 6/11 | 5/11 |

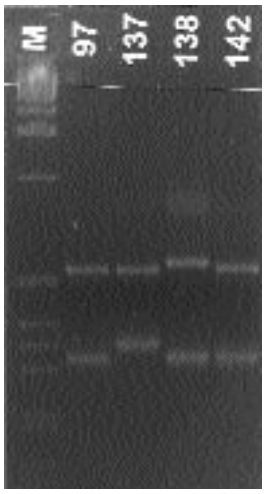
[a] Isolates sorted according to results with primers 5'CP/3'CP and *Msp*I restriction.
nt : not tested; -: no reaction.



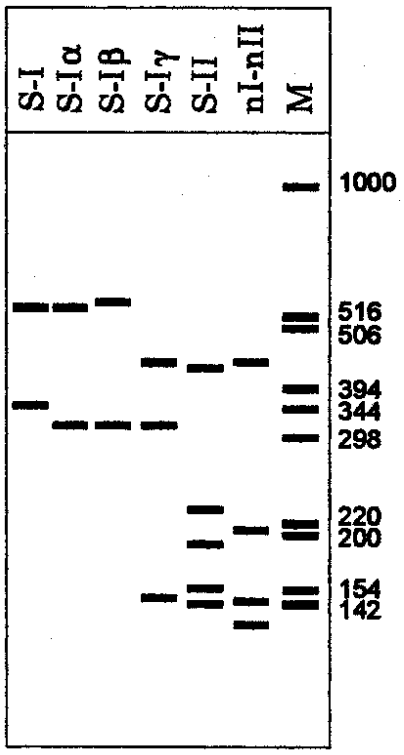

a




b




c




d

**Fig. 2a, b, c** and **d.** Electrophoretic analysis in 3% NuSieve 3:1 agarose of the amplified products obtained for some cucumovirus isolates with primers 5'CP /3'CP, digested with *Msp*I. Gels stained with ethidium bromide. M indicates molecular weight markers. Fig. 2d represents the different patterns found.

**Table 8.** Detection of cucumoviruses by ELISA variants.

| Virus isolate (RT no.)[a] | 5'CP/3'CP | DAS-ELISA Plate no. | | | | | | ACP-ELISA Plate no. | | | TAS-ELISA Plate no. | | | | | | | | | | | Positive[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 4 | 7 | 10 | 13 | 16 | 52 | 55 | 61 | 19 | 22 | 25 | 28 | 31 | 34 | 37 | 40 | 43 | 46 | 49 | |
| 3 | S-I | – | – | ++++ | + | ++++ | ++ | ++ | ++++ | + | + | ++ | +++ | + | + | + | – | ++ | ++ | – | +++ | 16 |
| 8 | S-I | – | – | ++++ | + | +++ | ++ | ++ | ++++ | + | + | ++ | +++ | + | + | + | – | ++ | ++ | – | ++ | 16 |
| 10 | S-I | – | + | ++++ | + | +++ | ++ | + | ++++ | + | + | ++ | ++ | + | + | + | | + | ++ | – | ++ | 17 |
| 51 | S-I | – | + | +++ | + | +++ | ++ | + | ++++ | + | + | ++ | + | + | + | + | – | + | ++ | – | + | 17 |
| 52 | S-I | – | – | ++++ | – | +++ | ++ | ++ | ++++ | + | ++ | ++ | +++ | + | + | + | – | ++ | ++ | – | + | 15 |
| 72 | S-I | – | + | +++ | – | +++ | ++ | ++ | ++++ | + | ++ | +++ | ++ | + | + | + | – | + | ++ | – | ++ | 16 |
| 86 | S-I | – | + | ++++ | – | +++ | ++ | ++ | ++++ | + | ++ | +++ | ++ | + | + | + | – | – | ++ | + | + | 16 |
| 87 | S-I | – | – | ++++ | + | ++++ | + | ++ | ++++ | + | + | ++ | ++ | + | + | + | – | + | + | – | – | 15 |
| 88 | S-I | – | – | ++++ | + | ++++ | + | ++ | ++++ | + | + | ++ | ++ | + | + | + | – | + | + | – | – | 15 |
| 89 | S-I | – | – | +++ | – | ++ | + | ++ | ++++ | + | + | ++ | + | + | + | + | – | + | + | – | + | 15 |
| 90 | S-I | – | – | ++++ | + | ++++ | + | + | ++++ | + | + | ++ | ++ | + | + | + | – | + | + | – | + | 16 |
| 99 | S-I | – | – | +++ | + | ++ | + | + | ++++ | + | + | ++ | + | + | + | + | – | + | – | – | + | 15 |
| 103 | S-I | – | – | ++++ | – | ++ | – | + | +++ | – | – | + | + | + | – | – | – | + | – | – | + | 9 |
| 109 | S-I | – | – | ++++ | + | ++++ | + | +++ | ++++ | + | ++ | ++ | ++++ | + | ++ | + | – | +++ | ++ | – | +++ | 16 |
| 110 | S-I | – | – | ++++ | – | ++ | + | ++ | ++++ | + | + | + | + | + | + | – | – | + | + | – | + | 14 |
| 135 | S-I | – | – | +++ | – | ++++ | + | + | ++++ | + | + | + | + | + | + | – | – | + | + | – | + | 13 |
| 137 | S-I | – | – | ++++ | – | +++ | + | + | ++++ | + | + | ++ | + | + | + | – | – | + | + | – | + | 14 |
| 141 | S-I | – | – | ++++ | + | ++++ | + | ++ | ++++ | + | + | + | + | + | ++ | – | – | + | + | – | + | 15 |
| 145 | S-I | + | – | ++++ | – | ++++ | + | + | +++ | + | + | + | +++ | + | + | + | – | + | + | – | ++ | 16 |
| 38 | S-Iα | – | – | ++++ | + | +++ | ++ | + | ++++ | + | + | ++ | ++ | + | + | + | – | + | ++ | – | ++ | 16 |
| 54 | S-Iα | – | + | ++++ | – | +++ | ++ | ++ | ++++ | + | + | ++ | ++ | + | + | + | – | + | ++ | – | + | 16 |
| 55 | S-Iα | – | + | ++++ | + | +++ | ++ | ++ | ++++ | + | ++ | ++ | ++ | + | + | + | – | ++ | ++ | – | + | 17 |
| 97 | S-Iα | – | – | ++++ | + | ++++ | – | + | ++++ | + | + | ++ | ++ | + | + | + | – | + | + | – | + | 15 |
| 107 | S-Iα | – | – | ++++ | + | +++ | – | + | ++++ | + | + | + | ++ | + | + | + | – | + | + | – | + | 15 |
| 142 | S-Iα | – | – | ++++ | – | ++ | + | + | +++ | + | + | + | ++ | + | + | + | – | + | + | – | ++ | 15 |
| 138 | S-Iβ | – | – | +++ | – | ++ | – | + | +++ | + | + | + | + | + | + | + | – | ++ | + | – | + | 14 |
| 144 | S-Iγ | – | – | +++ | – | ++ | – | + | ++ | – | + | + | + | – | – | + | – | + | – | – | ++ | 10 |
| 21 | S-II | ++ | + | ++ | ++ | ++ | + | +++ | +++ | + | ++ | +++ | – | + | ++ | ++ | ++ | – | +++ | + | ++ | 18 |
| 39 | S-II | ++ | + | ++ | + | ++ | + | +++ | +++ | + | ++ | +++ | – | + | ++ | ++ | +++ | – | +++ | ++ | + | 18 |
| 67 | S-II | ++ | + | + | + | + | + | + | + | + | ++ | +++ | – | + | + | + | ++ | – | ++ | ++ | + | 18 |
| 68 | S-II | ++ | + | ++ | + | ++ | + | +++ | +++ | + | ++ | +++ | – | + | ++ | ++ | +++ | – | +++ | +++ | ++ | 18 |
| 69 | S-II | ++ | + | + | + | + | + | + | ++ | + | + | +++ | – | + | + | + | ++ | – | ++ | ++ | ++ | 18 |
| 70 | S-II | ++ | + | ++ | + | ++ | + | + | +++ | + | ++ | +++ | – | + | ++ | ++ | +++ | – | +++ | +++ | ++ | 18 |
| 71 | S-II | ++ | + | + | + | + | ++ | ++++ | ++++ | + | +++ | +++ | – | + | +++ | ++ | +++ | – | +++ | + | ++ | 18 |
| 96 | S-II | + | | + | + | – | – | + | +++ | + | + | ++ | – | + | + | + | + | – | + | – | + | 14 |
| 101 | S-II | ++ | – | + | ++ | ++ | + | +++ | +++ | + | + | + | – | + | ++ | + | + | – | + | + | + | 17 |
| 102 | S-II | ++ | – | ++ | ++ | ++ | + | +++ | +++ | + | ++ | ++ | – | + | ++ | ++ | + | – | + | + | + | 17 |
| 136 | S-II | + | + | ++ | ++ | + | – | +++ | +++ | + | + | ++ | – | + | ++ | + | + | – | + | – | + | 16 |
| 139 | S-II | – | – | + | + | – | – | + | – | – | – | + | – | – | – | – | – | – | – | – | – | 4 |
| 146 | S-II | ++ | – | ++ | + | ++ | – | ++ | +++ | + | ++ | ++ | – | – | ++ | ++ | +++ | – | + | + | ++++ | 15 |
| 6 | nonI-nonII | + | + | ++++ | + | ++++ | ++ | ++ | ++++ | + | ++ | ++ | +++ | + | ++ | + | – | ++ | +++ | – | ++ | 18 |
| 48 | nonI-nonII | – | – | + | + | +++ | ++ | ++ | ++++ | + | ++ | ++ | +++ | + | + | + | – | ++ | ++ | – | ++ | 16 |
| 85 | nonI-nonII | + | + | +++ | – | +++ | +++ | +++ | ++++ | + | ++ | +++ | ++ | + | ++ | + | + | ++ | ++ | – | + | 18 |
| 140 | 140 | – | – | – | – | – | – | + | ++ | – | – | – | – | – | – | – | – | – | – | – | – | 2 |
| 100 | TAV | – | – | – | – | – | – | + | ++ | – | – | – | – | – | – | – | – | – | – | – | – | 2 |
| 104 | PSV | – | – | – | – | – | – | + | ++ | – | – | – | – | – | – | – | – | – | – | – | – | 2 |

[a] Isolates sorted according to results with primers 5'CP/3'CP and *Msp*I restriction.
[b] Total number of positive reactions (out of 20 possible) for each virus isolate.

**Table 9.** Summary of the detection of cucumoviruses by ELISA variants.

| Virus isolates[a] | DAS-ELISA | | | | | | ACP-ELISA | | | TAS-ELISA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plate no. | | | | | | Plate no. | | | Plate no. | | | | | | | | | | |
| | 1 | 4 | 7 | 10 | 13 | 16 | 52 | 55 | 61 | 19 | 22 | 25 | 28 | 31 | 34 | 37 | 40 | 43 | 46 | 49 |
| S-I (19) | 1 | 4 | 19 | 10 | 19 | 18 | 19 | 19 | 18 | 18 | 19 | 19 | 19 | 18 | 14 | 0 | 19 | 17 | 1 | 17 |
| S-Iα (6) | 0 | 2 | 6 | 4 | 6 | 4 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 0 | 6 | 6 | 0 | 6 |
| S-Iβ (1) | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| S-Iγ (1) | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| All S-I like (27) | 1 | 7 | 27 | 14 | 27 | 22 | 27 | 27 | 25 | 26 | 27 | 27 | 26 | 25 | 22 | 0 | 27 | 24 | 1 | 25 |
| S-II (13) | 12 | 8 | 13 | 13 | 11 | 9 | 13 | 12 | 12 | 12 | 13 | 0 | 11 | 12 | 12 | 12 | 0 | 12 | 10 | 12 |
| nonI-nonII (3) | 2 | 3 | 3 | 2 | 3 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 0 | 3 |
| 140 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100-TAV | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 104-PSV | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total (46) | 15 | 18 | 43 | 29 | 41 | 31 | 46 | 45 | 40 | 42 | 43 | 30 | 40 | 40 | 37 | 13 | 30 | 39 | 11 | 40 |

[a] Isolates sorted according to results with primers 5'CP/3'CP and *Msp*I restriction. In parenthesis, number of the isolates.

Recently others (Perry *et al.*, 1992; Wahyuni *et al.*, 1993; Ilardi *et al.*, 1995) have compared with several methods, a more limited number of isolates from a restricted geographic area or from a single crop, and others, using one technique only (Crescenzi *et al.*, 1993; Fraile *et al.*, 1997) have compared a large number of samples. The biology of CMV has been thoroughly investigated but some information is still missing, so that application of different techniques at the same time on a wide range of isolates can provide useful information for research, diagnosis, plant breeding and epidemiology.

For practical detection, ELISA variants based on both polyclonal and monoclonal antibodies are widely used. PCR amplification is becoming important for detection and differentiation of viruses (Huttinga, 1996). Studies of host plant reactions although now less in fashion, have always given useful information not obtainable by any other approach.

We found good agreement among the different methods tested for detection and differentiation of CMV isolates, their results complementing also each other. With the primer pairs 5'CP/3'CP we obtained the expected amplification of an 870 bp fragment with almost all isolates tested. After digestion with *Msp*I, the two electrophoretic patterns corresponding to S-I and S-II were found for 32 of the 43 CMV isolates tested. Of interest are the three new patterns named Iα, Iβ and Iγ found for 6, 1 and 1 isolates respectively, similar to but distinct from the typical S-I pattern. Recently Fraile *et al.* (1997) using RNase protection assays (RPAs) detected two different patterns (A and B) among S-I isolates from Spain, with the typical S-I pattern (A) present for 73% of the isolates. It is possible that our atypical Iα and Iβ patterns correspond to the S-I isolates giving an RPA B pattern, although 4 of our 6 Iα isolates came from the Far East (China and Taiwan), and 2 from France but none from Spain and the Iβ isolate came from Iran. Roossinck and Palukaitis (p. 356, in Palukaitis and Zaitlin, 1997), on the basis of sequence data, proposed a new S-I grouping for Asian isolates, designed S-IB, all other isolates being referred to S-IA.

We also found a new pattern called 'nonI-nonII' for 3 isolates (6, 48 and 85). They are, according to RNA-2 based primers and serology, probably closer to S-I than to S-II. With primers 5'CP/3'CP we also tested PSV and TAV, not done by Rizos *et al.* (1992) and we did not find the 870 bp fragment. Isolate 140 does not appears to be a CMV, but recalls PSV. The other primers derived from CMV RNA2 (LS2.2/LS0.8 and LS2.2/FNY1.8), although tested with fewer samples, differentiated between S-I and S-II isolates (see Table 6), in agreement with the results obtained with 5'CP/3'CP. This confirms that genetic reassortement is at most infrequent for CMV (Fraile *et al.*, 1997). The CMV1/CMV2 primers, also based on RNA2 were cucumovirus-specific, detecting both S-I and S-II, and also PSV and TAV.

**Table 10.** Host plant reaction of the cucumovirus isolates.

| Virus isolate (RT no.)[a] | Original host plant | 5'CP/3'CP | *C. pepo* | | *N. benthamiana* | | *N. glutinosa* | | *N. tabacum* | | *C. quinoa* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | loc | sys | loc | sys | loc | sys | loc | sys | loc | sys |
| 3 | cucumber | S-I | 3[b] | 3 | 2 | 3 | 1 | 2 | 2 | 2 | 3 | 1 |
| 8 | melon | S-I | 3 | 3 | 2 | 3 | 1 | 2 | 1 | 2 | 3 | 0 |
| 10 | watermelon | S-I | 3 | 3 | 1 | 2 | 2 | 2 | 1 | 2 | 3 | 0 |
| 51 | melon | S-I | 0 | 2 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 3 |
| 52 | squash | S-I | 3 | 2 | 2 | 2 | 0 | 3 | 2 | 3 | 3 | 0 |
| 72 | na[d] | S-I | 1 | 3 | 1 | 2 | nt[c] | nt | 1 | 2 | 2 | 0 |
| 86 | melon | S-I | 1 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 2 | 3 |
| 87 | melon | S-I | 1 | 3 | 1 | 2 | 0 | 1 | 1 | 2 | 3 | 0 |
| 88 | melon | S-I | 1 | 3 | 1 | 2 | 0 | 2 | 1 | 2 | 3 | 1 |
| 89 | melon | S-I | 2 | 3 | 1 | 2 | 1 | 2 | 1 | 1 | 3 | 1 |
| 90 | melon | S-I | 1 | 2 | 1 | 2 | 1 | 2 | 2 | 2 | 3 | 1 |
| 99 | na | S-I | 3 | 3 | 0 | 2 | nt | nt | 1 | 2 | 3 | 0 |
| 103 | pepper | S-I | 1 | 2 | 1 | 3 | nt | nt | 1 | 2 | 1 | 0 |
| 109 | melon | S-I | 0 | 3 | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 0 |
| 110 | melon | S-I | 1 | 3 | 1 | 2 | 0 | 2 | 3 | 2 | 3 | 0 |
| 135 | bean | S-I | 1 | 3 | 2 | 2 | 1 | 2 | 2 | 2 | 3 | 0 |
| 137 | zucchini | S-I | 3 | 3 | 2 | 2 | 3 | 2 | 3 | 2 | 3 | 0 |
| 141 | bean | S-I | 1 | 3 | 0 | 2 | 0 | 2 | 1 | 2 | 1 | 0 |
| 145 | zucchini | S-I | 2 | 3 | 1 | 3 | 3 | 3 | 1 | 1 | 2 | 0 |
| 38 | melon | S-Iα | 0 | 2 | 2 | 2 | 0 | 1 | 1 | 2 | 2 | 0 |
| 54 | cornflower | S-Iα | 1 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 3 | 1 |
| 55 | tomato | S-Iα | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 |
| 97 | cucumber | S-Iα | 2 | 3 | 2 | 3 | nt | nt | 0 | 1 | 3 | 0 |
| 107 | na | S-Iα | 2 | 2 | 2 | 3 | 0 | 2 | 1 | 2 | 2 | 0 |
| 142 | bean | S-Iα | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| 138 | bean | S-Iβ | 2 | 3 | 0 | 2 | 1 | 2 | 2 | 2 | 3 | 0 |
| 144 | bean | S-Iγ | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| 21 | zucchini | S-II | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 2 | 0 |
| 39 | squash | S-II | 0 | 3 | 1 | 0 | 0 | 2 | 2 | 0 | 2 | 0 |
| 67 | lettuce | S-II | 0 | 2 | 0 | 1 | 0 | 2 | 2 | 1 | 1 | 0 |
| 68 | spinach | S-II | 0 | 1 | 0 | 2 | 1 | 2 | 1 | 1 | 3 | 0 |
| 69 | spinach | S-II | 0 | 2 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 0 |
| 70 | pea | S-II | 1 | 3 | 1 | 2 | nt | nt | 1 | 1 | 2 | 0 |
| 71 | na | S-II | 2 | 3 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 0 |
| 96 | *Beta vulgaris* | S-II | 2 | 3 | 1 | 1 | nt | nt | 2 | 2 | 3 | 0 |
| 101 | *Vinca major* | S-II | 1 | 3 | 2 | 2 | nt | nt | 2 | 2 | 3 | 0 |
| 102 | pepper | S-II | 2 | 3 | 1 | 2 | nt | nt | 2 | 2 | 2 | 2 |
| 136 | carnation | S-II | 1 | 3 | 2 | 2 | 1 | 2 | 3 | 2 | 3 | 0 |
| 139 | bean | S-II | 0 | 1 | 1 | 2 | 0 | 0 | 2 | 1 | 1 | 0 |
| 146 | lupin | S-II | 0 | 0 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 0 |
| 6 | melon | nonI-nonII | 3 | 3 | 2 | 2 | 1 | 3 | 2 | 2 | 3 | 0 |
| 48 | squash | nonI-nonII | 0 | 3 | 1 | 2 | 1 | 2 | 3 | 0 | 3 | 0 |
| 85 | melon | nonI-nonII | 0 | 3 | 1 | 3 | nt | nt | 1 | 2 | 3 | 0 |
| 140 | bean | 140 | 0 | 0 | 2 | 2 | 1 | 0 | 2 | 1 | 2 | 0 |
| 100 | na | TAV | 0 | 0 | 0 | 2 | 1 | 2 | 1 | 2 | 2 | 0 |
| 104 | na | PSV | 0 | 0 | 0 | 2 | 1 | 2 | 1 | 2 | 2 | 0 |

[a] Isolates sorted according to results with primers 5'CP/3'CP and *Msp*I restriction.
[b] Symptom severity from 0 to 3.
[c] nt: not tested.
[d] na: not available.

**Table 11.** Summary of the host plant reaction of cucumovirus isolates.

| Virus isolates[a] | *C. pepo* | | *N.benthamiana* | | *N. glutinosa* | | *N. tabacum* | | *C. quinoa* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | loc | sys | loc | sys | loc | sys | loc | sys | loc | sys |
| S-I (19) | 17 | 19 | 16 | 19 | 11/16 | 16/16 | 18 | 18 | 18 | 6 |
| S-Iα (6) | 4 | 6 | 4 | 5 | 1/5 | 4/5 | 4 | 6 | 6 | 1 |
| S-Iβ (1) | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| S-Iγ (1) | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| All S-I like (27) | 22 | 27 | 21 | 25 | 13/22 | 21/23 | 24 | 26 | 26 | 7 |
| II (13) | 6 | 12 | 9 | 11 | 4/9 | 8/9 | 12 | 11 | 13 | 1 |
| non I-non II (3) | 1 | 3 | 3 | 3 | 2/2 | 2/2 | 3 | 2 | 3 | 0 |
| 140 (1) | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| TAV (1) | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| PSV (1) | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Total (46) | 29 | 42 | 34 | 42 | 22/36 | 34/36 | 42 | 42 | 45 | 8 |

[a] Isolates sorted according to results obtained with primers 5'CP/3'CP and *Msp*I restriction. In parenthesis, number of the isolates. Fractions indicate that not all the isolates were tested.

Among ELISA variants used in plant virology, DAS ELISA is the most common followed by TAS ELISA, whereas the use of ACP ELISA is sometimes problematic with field samples and with some plant species. In DAS ELISA the antisera we used displayed different specificities and those produced against S-II isolates were more specific than those produced against S-I, although this conclusion requires confirmation using a large number of antisera. With at least two antisera (plates 7 and 13) most isolates were detected. The tests confirmed that for cucumoviruses ACP ELISA is less specific than the other formats (compare plates 4 and 61 done with the same antiserum) and two of the ACP plates even detected PSV and TAV.

The Mabs used in TAS-ELISA showed different degrees of specificity. A few were specific for S-I (plates 25 and 40) or S-II (plates 37 and 46), while one (plate 22) detected all CMV isolates tested. In conclusion, the serological differentiation of S-I and S-II appeared reliable only using Mabs, but not using Pabs in DAS or in ACP-ELISA. Considering the presence of common epitopes found in ACP ELISA in all the cucumoviruses tested, it would be of interest to explore the possibility of selecting cucumo-specific Mabs, for use in a universal cucumovirus assay, like that employed for potyviruses.

The 3 isolates typed as 'nonI-nonII' by 5'CP/3'CP primers, can be classified as S-I with Mabs considering the positive reaction on plate 25 and the absence of reaction on plates 37 and 46; however results in other plates were not in total agreement. The subgroup-specific Mabs allowed classification of isolate 144 as S-Iγ, thus confirming PCR analysis, which considered it as S-I.

Isolate 140 cannot be considered a CMV isolate, according to the overall results of the Ringtest. However it does appear to be a cucumovirus since it was detected on plates 52 and 55, together with PSV and TAV.

Although infections on host plants were not checked by back inoculation or other methods, an impossible task considering the number of plants to be tested, our data can be considered reliable since latent infection of the hosts we used has never been reported. Most isolates infected zucchini, independently of the original host, and the symptoms were severe in most cases, confirming the importance of CMV for this crop. Only PSV, TAV, and isolates 140 and 146 did not systemically infect zucchini. In our conditions, test plants reactions could not be used to differentiate CMV isolates as already reported by Wahyuni *et al.* (1992). It is noteworthy that some isolates, mainly S-I, systemically infected *C. quinoa.*

What is the most appropriate type of assay for detecting and typing CMV? Although we did not compare the sensitivity of the different techniques, PCR is basically more sensitive than serological methods. However the results given by ELISA are generally satisfactory since CMV is usually found in high concentration in naturally infected samples.

RT-PCR is easily applied to typing CMV isolates, and use of 5'CP/3'CP primers followed by restriction can go beyond the classic subgrouping, revealing the presence of different clusters within subgroup I.

Choice of method finally depends on the number of samples to test (since ELISA is more suitable for large numbers of samples) on the laboratory facilities available, and the specific need for detailed typing.

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