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# Production of a Monoclonal Antibody Specific for Citrus Tristeza Virus

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*In this work Citrus Tristeza Virus (CTV) was purified from the bark and midrib tissue of infected plants by pulverization of frozen tissue samples in a mortar with extraction buffer, with polyethylene glycol (PEG), and by sucrose gradient centrifugation. In an alternative purification method, CTV was purified by Sephacryl 300 column chromatography after precipitation by PEG. A hybridoma line secreting monoclonal antibody (MAb) highly specific for the CTV was generated by fusion of splenic lymphocytes of immunized BALB/c mice with non-secreting mouse myeloma cells followed by selection in hypoxanthine-aminopterin-thymidine (HAT) medium, and finally, by screening culture fluids for CTV affinity in ELISA. The MAb produced by the CTV-IG8 hybridoma clone was highly specific to CTV and did not react with non-infected citrus plant extracts.*

**Keywords:** *Citrus Tristeza Virus, ELISA, immunodiagnosis, monoclonal antibody*

## INTRODUCTION

Citrus Tristeza Virus (CTV) is distributed worldwide and is the most important pathogen of citrus (Roistacher & Moreno, 1991). The virus comprises a wide range of biologically distinct isolates, and differs greatly in symptom expression. The characteristic prevalent symptom is incompatibility between scion and sour orange rootstocks. Decline and dieback, growth reduction, stem pitting, and yellowing of the plants are also induced in infected plants. There is evidence that CTV isolates are frequently populations from which variants with distinct properties can be selected and that also contain multiple defective RNAs (DRNAs). A disease phenotype may, therefore, result from a mixture of viral components that exist in different portions and co-replicate in the infected plants (Hılf *et al*., 1999). The virus symptoms depend on the virus isolates, environmental conditions, and citrus cultivars. CTV is a member of the phloem limited closterovirus group (Bar-Joseph *et al*., 1989). The genome of CTV consists of single stranded DNA. It is a monopartide, aphid transmitted virus with

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flexuous, filamentous particles 2000 nm long which contain 4000 molecules of a major 25 kDa capsid protein (Sekiya *et al*., 1991; Yang *et al*., 1999; Lopez *et al*., 2000). The antigenic structure of a virus particle built of several identical subunits of the capsid protein consists of a complex of two fundamentally different types of epitopes: linear, or continuous epitopes, are defined by a linear array of 6–8 amino acids. The epitopes require a certain native conformation (Lee *et al*., 1987; Van Regenmortel & Dubs, 1993). The antigenic structure and antigenic information are often used for practical purposes, such as the differentiation of virus isolates with different pathogenicity (Nikolaeva *et al*., 1997).

Çukurova is an important citrus production area in the Eastern Mediterranean Region of Turkey. In the region CTV remains as a potential threat for citriculture since the virus is constantly present with its vector and it is also epidemic in neighbouring countries.

Purification of antigen for the production of CTV-specific antiserum or monoclonal antibody (MAb) has been difficult since CTV is a phloem-limited virus with a very narrow host range confined to woody perennials. In this paper, we report the purification of CTV from bark and midrib of infected citrus plants by different methods, and preparation of antiserum against CTV as well as the development of a hybrid cell (IG-8) producing a MAb specific to CTV with the ultimate aim of developing an efficient immunodiagnostic system.

## MATERIALS AND METHODS

### Virus Isolates

Eight CTV infected isolates, Turkish isolates (Iğdır, Serdengecti, Kazanlı, Dörtyol, İzmir), Cyprus isolate (Kıbrıs) and two different American isolates (514 and 519) and non-infected control plant tissues were used for the study. The samples from orchards of citrus were taken, tested by ELISA and infected trees were determined. However, Iğdır isolate was only used for purification of CTV. The isolates were transmitted to Etrog citron (*C. medica* L.) seedlings to multiply the virus.

### Antibody

CREC35 (Florida) (SANOFI) polyclonal antibodies (PAbs) are used in the detection of CTV isolates.

The biochemicals and immunochemicals were purchased from Sigma, Boehringer Mannheim or Calbiochem. The culture medium DMEM as well as supplements such as sodium pyruvate, L-glutamine and antibiotics were purchased from either Flow or Gibco. Fetal calf serum (FCS) was a product of Flow. Plasticwares were obtained from NUNC and hybridoma subisotyping kit from Calbiochem. The non-secreting myeloma line FO (ATCC CRL 1646) was obtained from the American Type Culture Collection.

### Assays

*Virus Isolates and Propagation Hosts.* Iğdir isolates of CTV were used for purification, and different isolates were used for ELISA. The Iğdir isolate was transmitted to Etrog citron (*C. medica* L.) seedlings to multiply the virus.

*Purification of CTV.* CTV virions were sized fractioned by sucrose gradient centrifugation and also according to Lee et al. (1988).

The purification of CTV was performed with respect to the procedure described by Bar Joseph *et al*. (1985) and Lee *et al*. (1988) with some modifications. Frozen powder of bark and midrib of infected citrus plants was ground with liquid nitrogen in a mortar with a pestle, and was thawed and homogenized in the extraction buffer after PEG precipitation. The pellet was resuspended and then layered into a column for gel filtration on a $1.5 \times 70$ cm water jacketed column of Sephacryl 300 (S 300) equilibrated with 0.05 M-Tris-HCl, pH 7.8, 1

mM-MgCl, 0.2%-Na azide (separation buffer). The fractions of 1 ml were tested by an indirect ELISA system. Briefly the plates were coated with 50 µl of 1/10 diluted fractions. The virus particles were detected by anti-CTV (GREC 35) PAbs. Alkaline phosphatase-labelled goat anti-mouse IgG (for monoclonal anti-CTV) or anti-rabbit IgG (for polyclonal anti-CTV) were used as the conjugate. As a control, the plates were coated with fractions of healthy citrus extract after gel filtration on a S 300 Sephacryl column under the same conditions.

*Serology.* The dot blot method was also used for the detection of CTV in the plant tissues. A drop of sample from infected or non-infected plant tissue was added onto a polyvinyl difluoride (PVDF) membrane (Immobilon-P, Millipore) and dried at room temperature. The blotted membrane was incubated for 1 h with CTV specific antibody in blocking buffer (1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS], i.e. 10 mM-Na phosphate, pH 7.2, 0.9% NaCl) containing 0.05% Tween-20. The membrane was washed in PBS two times for 10 seconds and was incubated for 30 min with a secondary antibody diluted in blocking buffer containing 0.05% Tween-20. The membrane was washed in PPS two times for 10 s. Bound antibody was visualized using the NBT/BCIP colorimetric substrate system (Promega). The blot was then washed in distilled water to stop the reaction.

*Electron microscopy.* 10 µl of CTV particles purified by chromatography on Sephacryl S 300 were placed on a carbon coated grid (Milne & Luisoni, 1977) and examined under an electron microscope (JEOL-JEM100C TEM).

*Immunization.* Eight-week-old BALB/c mice were immunized subcutaneously with 54 µg CTV emulsified in complete Freund's adjuvant (CFA) in 0.1 ml. Two and five weeks after the initial injection, the animals received subcutaneous injections of 54 µg CTV emulsified in incomplete Freund's adjuvant (IFA) in 0.1 ml. After three weeks, the animals were given three intravenous injections of 54 µg of CTV on three consecutive days (Garver *et al*., 1988).

*Fusion.* The spleen cells were isolated from the mouse with the highest anti-CTV antibody titre on day 59 and were fused with mouse myeloma cells F0 (ATTC CRL 1646) at 1/6 ratio. The standard fusion, cloning and subcloning protocols were performed as described previously (Galfre & Milstein, 1981). Polyethylene glycol 4000 (Fluka) (PEG) was used as fusion agent.

*ELISA.* The indirect and sandwich ELISA methods were used to detect antibody activity in the mouse sera and hybridoma supernatant or the reactions of different isolates with IG-8 (Engwall, 1980; Medina, 1988).

In the direct ELISA system, the anti-CTV MAb conjugated to AP was used (Schots *et al*., 1988).

The competitive ELISA system was used to detect CTV in different CTV isolates. 100 ng CTV (Iğdır isolate) in 100 µl PBS to the wells of the ELISA plate. The plate was incubated overnight at 37°C. Different CTV extracts (100 µl) were mixed with 5 µl IG-8-AP conjugate and transferred to ELISA micro wells coated with 54 ng CTV. Inhibition of coated CTV binding to IG-8 was determined from results (Jenny *et al*., 1993). As control, 0.01–10 µl CTV in 0.1 ml PBS healthy plant extract was used.

*Determination of Ig type.* The heavy chain type of MAb was determined with an Ig subisotyping kit (Behring Diagnostic, CA, USA).

*Purification of MAb.* The antibody was precipitated from hybridoma culture supernatant at 40 percent $(NH_4)_2SO_4$ saturation, in PBS (150 mM-potassium-phosphate, pH 7.2) and dialysed against the same buffer and applied to a Protein G MAb Trap kit (Jeanson *et al*., 1988). Mouse MAb was eluted with 1 M-glycine buffer, and the fractions were immediately neutralized.

*Conjugation.* 0.35 mg of AP (Boehringer Manheim, Germany) in a 0.035 ml volume, was treated with 0.001 ml of 25% glutaraldehyde solution. After incubation for 50 min at 25°C, 0.15 mg of IgG (affinity purified) in potassium phosphate buffer was added. The mixture was gently stirred for 75 min at 25°C, then transferred on to Sephacryl S 300 (Sigma) column and the conjugate was eluted with Tris-HCl buffer (50 mm, pH 8.0, containing 0.1 M-NaCl, 1 mM-$MgCl_2$, 0.1% Na azide (w/v)) in fractions of 1 ml (Öztürk, 1997).

## RESULTS

### Detection of CTV Infected Trees

Trees showing quick decline or sudden dieback symptoms and with low starch in the roots were suspected for tristeza. A section of bark removed from the bud-union area of a tristeza-infected tree will usually show inverse pitting on the inner surface of the bark. This symptom is highly diagnostic for tristeza. CTV was detected in infected citrus extracts by dot-blotting ELISA (Figure 1).

### Purification of CTV Via Sucrose Gradient Fractionation

CTV positive and negative isolates were size-fractionated by sucrose density gradient centrifugation and the virus concentration in each fraction after sucrose gradient fractions of CTV positive and negative plant extracts were compared for binding to anti-CTV PAbs by indirect ELISA (Figure 2).

The CTV particles were separated from citrus plant cell components by S 300 column chromatography. The ultraviolet adsorption spectrum of fractions at 260 nm showed a distinct peak in fractions 29–36. The same peak was not observed with the control (Figure 3). The ratio of 260/280 was 1.20.

Each fraction was assayed by ELISA. It was determined that the reaction was higher in the fractions of 29–36 (Figure 4).

### Electron Microscopy

The polyclonal antiserum was used to determine the virus particles. The antiserum reacted along virus particles in purified material and the flexuous particles are seen in the preparation (Figure 5).

### Preparation of MAb Anti-CTV

The mice with the highest titre of anti-CTV antibody were sacrificed and the spleen cells were used in the fusion. On the 16th day, the culture fluids were tested for the presence of anti-CTV by indirect ELISA. Of 259 original wells, 21 exhibited macroscopic visible hybridoma

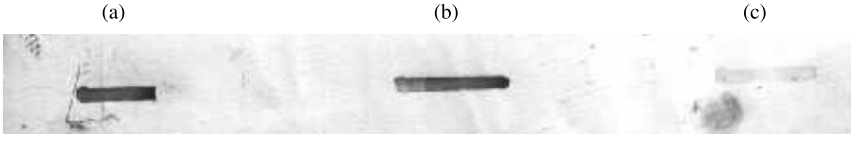


FIG. 1. Detection of CTV on membrane. (a) Iğdır; (b) Serdengeçti; (c) healthy citrus extract.

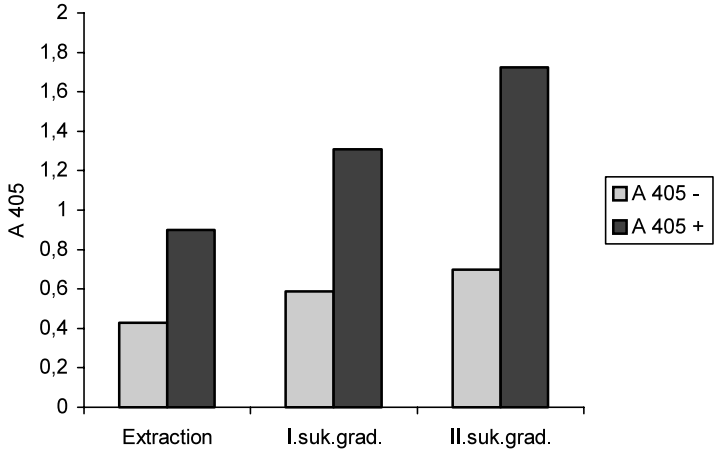


FIG. 2. Comparison of CTV infected ($A_{405}+$) and healthy ($A_{405}-$) samples after second sucrose gradient fractionation for activity by indirect ELISA.

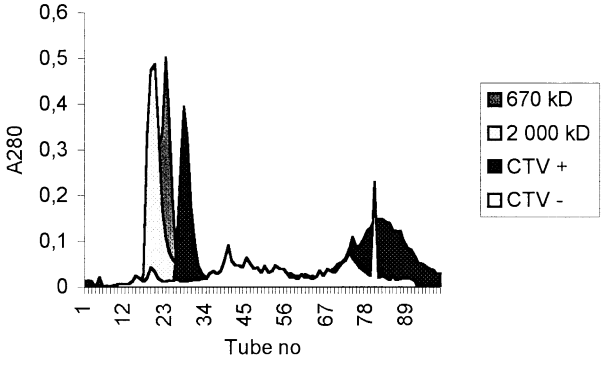


FIG. 3. Virus concentration peaks of infected and healthy samples.

clones in culture. Only 1 of the 21 produced anti-CTV antibodies and IG-8 was subjected to three subcloning steps by limiting dilution.

**Characterization of MAb Anti-CTV**

ELISA was used in screening of hybrid supernatants for the presence of antibody specific for CTV. Out of the positive hybrid clones, one (IG-8, producing antibody with the highest specificity) was subjected to three subsequent subcloning steps by limiting dilutions.

The MAb IG-8 was found to be of IgM/κ subisotype using a hybridoma subisotyping kit (Tables 1 and 2). MAb IG-8 was purified by $(NH_4)_2SO_4$ precipitation between 0–40% saturation and subsequent chromatography on protein A-Sepharose 4B. It was shown (Figure 6) that the second protein peak (absorbance 280 nm) was reactive with CTV. Immunoaffinity purified IG-8 was conjugated to AP. MAb IG-8-AP conjugate was purified on a Sephacryl 300 column. The main conjugate peak fractions were pooled and compared in regard to their specific activity in the ELISA. Figure 7 shows that the binding of anti-

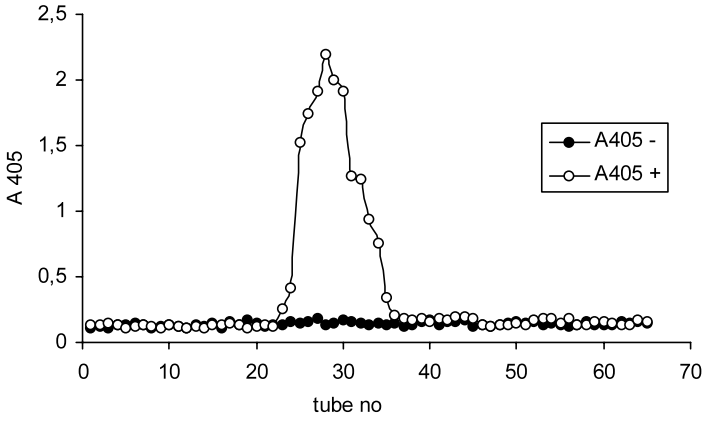


FIG. 4. Detection of CTV activity at the infected ($A_{405}+$) and healthy ($A_{405}-$) plant extracts after S 300 column chromatography. Fractions were coated (1:10) to the wells. Activity of CTV in the fractions were detected with polyclonal anti-CTV GREC 35.

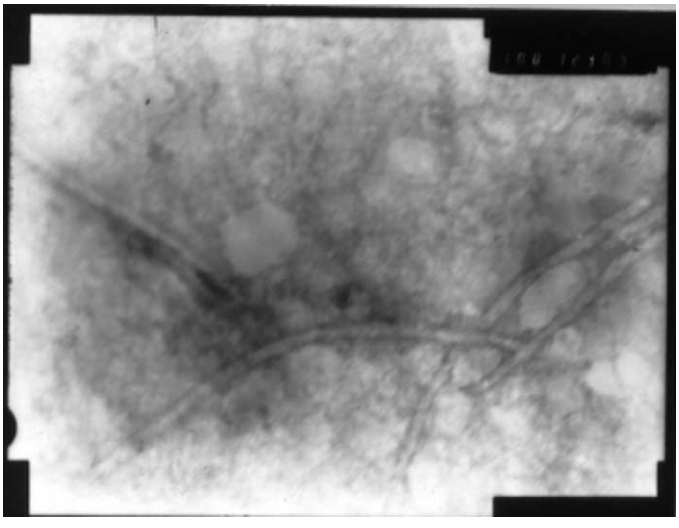

FIG. 5. Particles of CTV ( $\times$ 100 000).

CTV-AP to CTV is directly proportional to the amount added in the ELISA system. The purified conjugate was separated from free IG-8 or AP.

The CTV in infected plant extracts was determined by their competition with the free CTV for binding to IG-8. As shown in Figure 8, inhibition increased due to CTV concentration.

## DISCUSSION

Pure material is essential for further characterization and differentiation of virus from other viruses and to carry on serological work. Purification of the flexuous type of virus has special problems because of the aggregation, shearing, absorption to host membranes and lower concentration (Lee *et al*., 1987). CTV was purified simply by using $Cs_2SO_4$-sucrose cushion

TABLE 1. Reactions of different isolates with IG-8

| | Sandwich ELISA | Indirect ELISA |
|---|---|---|
| Iğdır | + | + |
| Serdengeçti | − | + |
| Kazanli 5 | − | − |
| İzmir H3 | − | − |
| Dörtyol | ± | − |
| Kıbrıs | − | + |
| 519 | − | − |
| 514 | − | − |
| Healthy citrus (control) | − | − |

TABLE 2. Detection of immunoglobulin heavy and light chains of IG-8

| Subisotype | $A_{405}$ |
|---|---|
| IgG | 0.588 |
| $IgG_1$ | 0.464 |
| $IgG_{2a}$ | 0.538 |
| $IgG_{2b}$ | 0.467 |
| IgA | 0.495 |
| $IgG_3$ | 0.499 |
| IgM | 2.214 |
| λ (light chain) | 0.430 |
| κ (light chain) | 1.666 |

step gradient and virus concentration was increased with the reduction of centrifugation time (Bar-Joseph *et al*., 1985). The yield of virus was also increased with the additive (polyethylene glycol *p*-isooctylphenyl ether (PGIE) in the extraction buffer, the PEG precipitation step and $Cs_2SO_4$ gradient centrifugation (Van Regenmortel & Dubs, 1993). Finally, a Bio-gel A-15 column was also used for further purification of CTV (Lee *et al*., 1988).

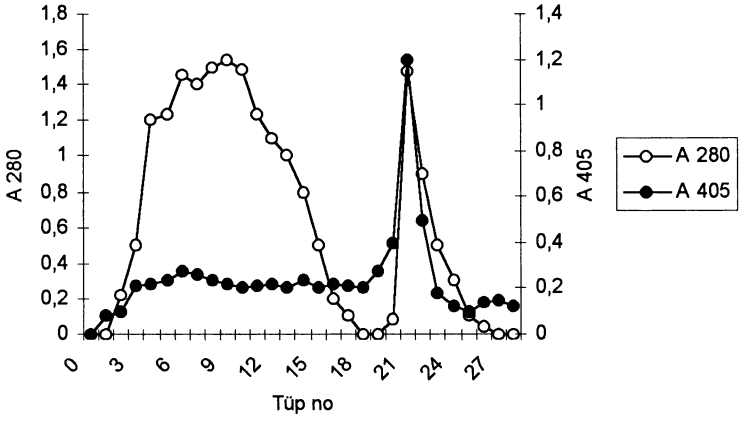


FIG. 6. Purification of MAb IG-8 on Protein G mabtrap kit.

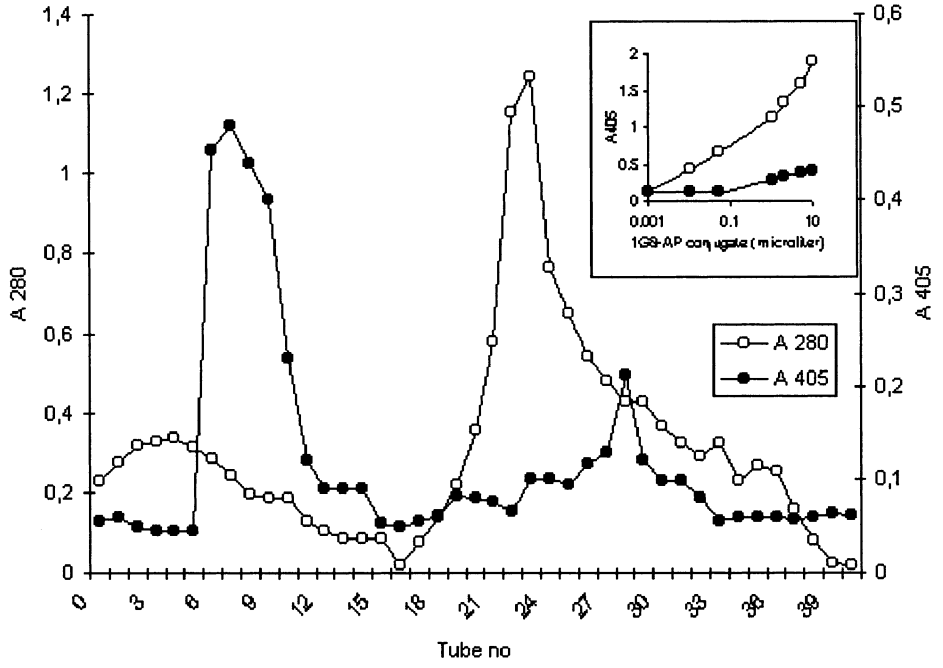


FIG. 7. Determinating of main conjugate peak on S 300 column and its ELISA activity ($A_{405}$). The first protein peak containing fractions ($A_{280}$) that contain the purified conjugate was combined. Small pictures shows the determination of purified anti-CTV-AP conjugate activity at infected (-○-) or healthy (-●-) plant extract coated plate (with direct ELISA).

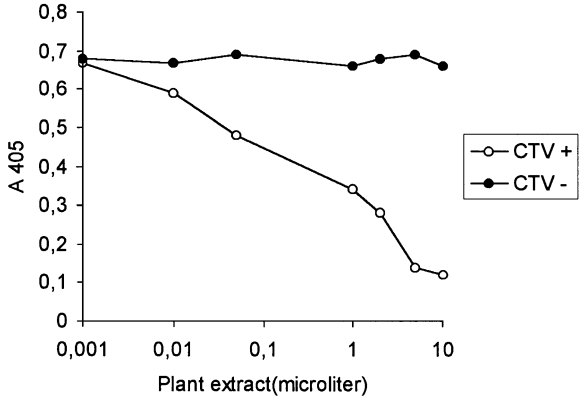


FIG. 8. Determination of free CTV inhibition in infected (CTV+) and healthy (CTV−) plants.

It appears that using PEG precipitation followed by Sephadex 300 column chromatography is a convenient method for further CTV purification The virus yield was 454 μg $ml^{-1}$ and used successfully for MAb production. The results have indicated that column chromatography is a suitable alternative method to be used for further purification of CTV under our conditions.

This study aims to develop a functional antibody against CTV. The detection of CTV in infected citrus isolates can be most useful in the early diagnosis and treatment of infection with CTV.

Most commercial CTV tests are based on PAbs. Unless a specific test for isolates from Turkey, these tests are affected by a large proportion of false positive results. Thus, the sensitivity has been sacrificed yielding many false negative results.

In this study, we developed a hybridoma clone IG-8 producing antibody highly specific to Citrus Tristeza Virus in Iğdır, Serdengeçti and Kıbrıs isolates. The tests performed have shown that the MAb IG-8 obtained by hybridoma technology has a high specificity for CTV. ELISA tests have shown that the isolates Serdengeçti and Kibris could produce a single epitope specific for IG-8 while the Igdir isolate has more than one. This result suggests that the antibody described can be used for the immunodiagnosis of CTV in isolates from Turkey.

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