Direct Detection of Molluscum Contagiosum Virus in Clinical Specimens by Dot Blot Hybridization

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A dot blot hybridization protocol was developed for the direct detection of molluscum contagiosum virus (MCV) DNA in clinical specimens submitted for virus isolation. Samples were concentrated by high-speed centrifugation and treated with proteinase K; this was followed by a single phenol-chloroform extraction step. The DNA was denatured, and the entire volume was spotted onto a nitrocellulose membrane. A biotinylated DNA probe specific for the BamHI-C region of MCV type 1 was used for hybridization. Evidence of MCV DNA was visualized by using streptavidin alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium as the substrate. Results showed that nonspecific hybridization does not occur with herpes simplex virus- or orf virus-infected clinical specimens and that dot blotting is more sensitive and reproducible than electron microscopy.

Molluscum contagiosum virus (MCV) infection is generally a self-limiting benign disease that produces single or multiple lesions on most skin areas. The disease has a worldwide distribution, and most healthy humans are susceptible (16). Although the lesions usually resolve spontaneously, aberrant forms have been described, especially in association with immunocompromised patients such as those with AIDS (2, 9, 16).

Since a differential diagnosis is often necessary to exclude other malignant and nonmalignant diseases, skin scrapings, swabs, or both are commonly submitted to the laboratory for virus isolation studies (2, 14, 15, 18). It is in the clinical virology laboratory that MCV presents a unique challenge to the microbiologist. On the one hand, the lesions are often pathognomonic to the attending physician. If some doubt exists, then formalin-fixed lesions can be embedded in paraffin, sectioned, and examined by histological or immunohistochemical methods (13). A characteristic cytopathology, consisting of giant cells, each with a large intracytoplasmic hyaline acidophilic granular mass known as a molluscum body, is usually present and is easily identified by the pathologist or cytotechnologist (6). On the other hand, this organism cannot be definitively identified in the virus laboratory by standard methods because, at present, MCV cannot be propagated in any known cell line. It does produce an abortive infection in vitro with slight cytopathic effects in some cell lines (16). However, it does not produce infectious progeny (10). Therefore, routine immunohistochemical methods have been unsuccessful in the identification of MCV in cell culture.

As a member of the Poxviridae family, this organism produces large numbers of characteristic poxlike virions in the skin lesion (17), and this has facilitated their presumptive identification by electron microscopy (EM). However, there are some limitations and not all laboratories have this capability. Therefore, the development of a dot blot hybridization procedure with a biotinylated DNA probe produced from an MCV recombinant plasmid (3) would fill this need for a test procedure that would detect and definitively identify MCV in clinical samples submitted for routine virus isolation studies.

MATERIALS AND METHODS

Clinical specimens. Swab or skin scrapings were collected for virus isolation and stored in virus holding medium (Hanks balanced salt solution with 0.5% gelatin [pH 7.0]) at −70°C. Some scrapings were from our diagnostic file and others were generously provided by Ann Warford of Southern California Kaiser Permanente Regional Virology Laboratory.

The specimens used in this study were (i) culture negative for herpesvirus but showed cytopathic effects resembling that of MCV or (ii) from patients whose clinical symptoms were suggestive of MCV. A portion of each specimen was tested by EM for the presence of virus particles. Our standard EM procedure has been described previously (11). Nonspecific controls consisted of clinical specimens that were confirmed by culture or EM to be infected with herpes simplex virus type 1 or 2 or orf virus.

DNA biotinylated probe. The 13.5-kb BamHI-C fragment of the MCV type 1 (MCV-1) genome was ligated into the 3.6-kb pAT153 plasmid vector (3, 21). This constructed recombinant plasmid has been designated pMCV-1-B-C. The competent HB101 strain of Escherichia coli was transformed with this plasmid by standard methods in the California State Department of Health Services laboratory, and the DNA was subsequently purified by multiple phenol extractions following established procedures (19), with slight modifications. The biotin-labeled probe was prepared from this purified plasmid by nick translation (ENZO Diagnostics, Inc., New York, N.Y.) by following the protocol of the manufacturer.

Dot blot. DNA was concentrated and extracted from clinical specimens in microfuge tubes as follows. Thawed specimens were vortexed, and then portions of up to 1.2 ml were centrifuged in polyallomer microcentrifuge tubes.

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(Beckman, Fullerton, Calif.) at 88,000 \times g for 30 min at 4°C. This volume was the maximum capacity of these particular tubes. The volumes of the original specimens were generally less than or equal to 2.5 ml. The pellets were dissolved in 400 \mu l of protease K buffer (1 mg of protease K per ml, 0.1 M Tris hydrochloride [pH 7.5], 2% sodium dodecyl sulfate [SDS], 20 mM EDTA) by incubating them at 45 to 50°C for 30 min. Approximately 1 \mu g of salmon sperm DNA was then added and the solution was mixed thoroughly.

A phenol extraction was done in the same tube by using approximately 0.9 ml of premixed phenol-chloroform solution (AMRESCO, Inc., Solon, Ohio). Residual phenol was removed by a single chloroform extraction step. The aqueous layer was collected into a clean microcentrifuge tube, and the DNA was denatured by the addition of a 1/10th volume of 4 N NaOH and was incubated at 72°C for approximately 30 min. After cooling to room temperature, this mixture was neutralized by the addition of an equal volume of 2 M ammonium acetate.

This entire volume (approximately 0.5 ml) was then spotted onto an Immobilon-NC nitrocellulose membrane (Millipore Corp., Bedford, Mass.), which was presoaked in 1 M ammonium acetate, using a vacuum apparatus (Bethesda Research Laboratory, Gaithersburg, Md.). After rinsing briefly in 1 M ammonium acetate, the membrane was air-dried and baked for 1 to 2 h under vacuum at 80°C (8). Positive and negative controls were made by spotting known amounts of pMCV-1-B-C and salmon sperm DNA, respectively. Each control was subjected to alkaline denaturation before use, as described above.

Positive and negative control spots consisting of 50 ng of pMCV-1-B-C DNA and 500 ng of salmon sperm DNA, respectively, were denatured as described above and were included in every dot blot run.

Hybridization. Membranes were prehybridized in a solution (final concentrations are given) of 50% deionized formamide, 5× SSC (750 mM sodium chloride, 75 mM sodium citrate), 5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrroldione, 0.1% bovine serum albumin), 25 mM sodium phosphate buffer (pH 6.5), 0.5% SDS, and 0.5 mg of denatured salmon sperm DNA per ml at 43°C for 3 to 4 h.

These membranes were then hybridized overnight at 43°C in a probe solution (final concentrations are given) of 47% deionized formamide, 5× SSC, 5% dextran sulfate, 1× Denhardt solution, 20 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 0.2 mg of denatured salmon sperm DNA per ml, and 200 to 300 ng of denatured biotinylated probe per ml. The optimal probe concentration was previously determined by titration. DNA was denatured by heating it in boiling water for 10 min (7).

Each membrane was washed twice at room temperature in 2× SSC with 0.1% SDS for 5 min each time, twice in 0.2× SSC with 0.1% SDS for 5 min each time, and twice in 0.16× SSC with 0.1% SDS at 55 to 60°C for 15 min each time. After a brief rinse in 0.2× SSC, they were soaked in blocking buffer (1× phosphate-buffered saline, 3% bovine serum albumin, 0.05% Triton X-100, 5 mM EDTA) at 36°C for 30 min. They were then reacted with streptavidin conjugated with alkaline phosphatase (DETEK-1-alk; ENZO Diagnostics) by following the instructions of the manufacturer. This was followed by five room-temperature washing steps in buffer (10 mM potassium phosphate buffer [pH 5.5], 0.5 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 2% bovine serum albumin) for 5 min each time and two rinses in predetection buffer (100 mM Tris hydrochloride [pH 8.8], 100 mM NaCl, 5 mM MgCl₂) for 2 min each time at room temperature. A solution containing 37 \mu g of nitroblue tetrazolium and 25 \mu g of 5-bromo-4-chloro-3-indolyl phosphate in predetection buffer was used as the enzyme substrate. A positive hybridization reaction was indicated by the appearance of a blue color.

RESULTS

The probe sensitivity limit was determined by multiple testing of different lots of probe. Each was hybridized with known amounts of pMCV-1-B-C DNA. As illustrated in Fig. 1, the endpoint was approximately 5 pg in every test. At this concentration the color was faintly visible. On the basis of the relative sizes of the plasmid vector and its insert, nearly 74% of the total DNA in each spot was BamHI-C. Therefore, the actual sensitivity limit of our probe with MCV DNA extracted from clinical samples was probably 7 to 10 pg. The negative control spot was included to ensure probe specificity.

Figure 2 shows the results of the probe specificity tests by using clinical specimens with confirmed herpes simplex virus or orf virus infections. Nonspecific hybridization did not occur with any of these samples. The negative and positive controls were included to further ensure the specificity and sensitivity of our probe.

Several comparative studies were done by using clinical specimens previously tested by EM to evaluate the detectability of the dot blot hybridization probe. Dot blots generally paralleled the EM results. Figure 3 is a composite of several experiments and shows relative differences of hybridization reactions with EM-positive and -negative specimens. There have been no false-negative dot blots observed to date.

DISCUSSION

The ability to provide an accurate identification of MCV, a potential pathogen, in a timely fashion could be very useful
in many clinical virology laboratories, particularly those lacking adequate cytology and EM facilities. It can be especially helpful in large facilities that function as reference laboratories or that handle requests and problems that are beyond the scope of smaller local laboratories.

EM examination of specimens submitted for virus isolation has been very successful in the presumptive identification of MCV, because this poxvirus produces large numbers of large characteristic particles in situ (1, 17). Not all laboratories have this capability; in those that do, the test has the following two major limitations. (i) Some poxviruses may share similar features at certain stages in their development and, therefore, may not be distinguishable by morphology alone (10). (ii) Assay sensitivity and precision are severely limited because there must be a minimum number of particles present in the sample (i.e., approximately 10^6/ml) before one can reproducibly locate them by EM techniques (5). A poorly collected sample may have far fewer particles; therefore, a negative EM result does not necessarily rule out the presence of MCV.

The probe sensitivity data presented here were the result of multiple tests. The logarithmic dilutions of MCV target DNA were used for convenience, recognizing that this limited the test’s precision. A more precise study with twofold dilutions above and below the 5-μg amount is needed. For these reasons, we can only estimate the sensitivity limits of this probe at this time. However, it demonstrates that a dot blot hybridization protocol can be more sensitive and, therefore, more reproducible than standard EM techniques, because as little as 10 pg of genomic DNA per sample can be detected. This indicates a capability of detecting as few as 5 x 10^4 virus particles per ml. The minimum number of particles per milliliter required for EM testing is equivalent to about 200 pg of genomic DNA per sample. Thus, the weakly reactive dot blots from EM-negative specimens are most likely true positives and reflect a relatively low number of virus particles in the clinical sample. During the course of this study, multiple EM tests were occasionally required on a single specimen to establish the presence of poxlike particles, whereas these same specimens always produced a strong positive dot blot.

The overall sensitivity of this procedure is a function of both probe sensitivity and methodology. Therefore, a protocol was designed to maximize viral DNA recovery by working with minimum volumes in the fewest possible steps. Thus, the initial centrifugation, proteinase K treatment, and DNA extraction steps were all carried out in the same microcentrifuge tube. Likewise, the final alkaline denaturation and neutralization steps were done in a single tube.

Ultracentrifugation was used primarily for convenience. A microcentrifuge (e.g., Eppendorf microcentrifuge) that generates approximately 16,000 x g could be substituted, provided that the centrifugation time was extended.

Probe specificity was a primary concern. Parr and associates (12) originally described three types of MCV based on their Bacillus subtilis restriction endonuclease analysis of the genome. Subsequent investigators identified only two distinct groups, which have been designated types 1 and 2 (3, 15). A marked heterogeneity of DNA restriction endonuclease cleavage patterns among isolates of the same subtype suggests significant strain variation, but Southern blot hybridization has consistently shown a high level of relatedness between MCV-1 and MCV-2 (20). On the basis of these observations, we were hopeful that this probe, which is derived from the BamHI-C fragment of MCV-1, would have sufficient homology to detect all or most strains in both subtypes. Our data, which to date have shown a direct correlation between EM-positive specimens and a positive dot blot, validate this probe’s utility in a test that is designed to detect MCV in most but not all properly collected specimens. Although the numbers are small, the panel of clinical specimens tested presumably reflects a mixture of types 1 and 2, because there is no preferred localization exhibited by either type (15, 20). Nonspecific hybridization was not observed with other viral or human DNA present in the clinical specimens. Herpes simplex and orthopoxviruses were used as nonspecific controls for two reasons. The first is that one of the major routes of MCV infection, especially in young adults, is believed to be by sexual transmission (6, 16). Therefore, specimens are often received from sexually transmitted disease clinics (4). Since herpesviruses are commonly isolated from these samples, it was important to rule out nonspecific reactions with these organisms. Second, we wanted to rule out cross-hybridization with other members of the Poxviridae, and orthopox virus is a member that can cause skin lesions in humans (6).

Our current probe has two inherent limitations. These will be addressed in future studies by improvements in probe design. For example, the plasmid vector will be removed prior to biotinylation. This will eliminate the potential of nonspecific hybridization of the vector sequences with extraneous DNA in the specimens. Another potential improvement could be realized by incorporating DNA sequences from both MCV-1 and MCV-2 into a pooled probe. This would maximize sensitivity by minimizing any adverse effects of type or strain variation on the dot blot hybridization assay described here. In addition, studies are in progress to explore other means of validating those specimens that are positive by this assay yet negative by EM.

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REFERENCES