Heterogeneity Among Strains of Mycoplasmatales virus-laidlawii 2

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SUMMARY

Five isolates of Mycoplasmatales virus-laidlawii 2 (MV-L2) derived from bovine strains of Acholeplasma laidlawii were shown to differ in host range, plaque morphology and neutralization tests with MV-L2 antiserum. Cross-testing using virus resistant clones of A. laidlawii confirmed the heterogeneity of this group. Adaptation of viruses to sub-optimal hosts was demonstrated.

The viruses so far isolated from and propagated in strains of Acholeplasma laidlawii fall into three major groups, namely Mycoplasmatales virus-laidlawii 1 (MV-L1), MV-L2 and MV-L3. The MV-L1 group comprises particles that are morphologically indistinguishable, but are not identical in their biological or serological properties (Gourlay, 1974; Maniloff, Das & Liss, 1974). MV-L2 viruses are sensitive to heating at 60 °C for 30 min and to treatment with detergents. They are spherical, 50 to 100 nm in diam., enveloped, and can be inhibited by homologous antiserum.

Several MV-L2 type viruses were isolated from strains of A. laidlawii in this laboratory, and were observed to vary in their plaque morphology and titre on different host strains. This paper reports their isolation and differences in their properties as observed by biological markers and serum neutralization tests.

Bovine isolates of A. laidlawii were received from Dr R. H. Leach of the Mycoplasma Reference Laboratory, Public Health Laboratory Service, Colindale, London N.W. 9, and strains M/1305/68 and BN1 from Dr R. N. Gourlay of the Institute for Research on Animal Diseases, Agricultural Research Council, Compton, Berkshire. Each strain was cloned by the recommended method (Subcommittee, 1972). Low passage levels were maintained by storage on agar blocks at -20 °C. For use, 48-h ‘sloppy agar’ cultures (Edward, 1947) were prepared and kept at +4 °C, subcultures being made as required.

Virus isolates in the series V1 to V2 were derived from lawns of apparently normal strains of A. laidlawii which had been washed by the method of Gourlay & Wyld (1972). These washes produced plaques on various indicator strains of A. laidlawii. Cloned virus was prepared by three cycles of plaque purification. The titre of each virus was built up and the final wash lyophilized with the addition of 20 % skimmed milk, and stored at -20 °C. At each stage of the cloning procedure, control washes of the indicator lawns were tested for virus release using strains 0-305, M/1305/68, 0-216 and BN1 of A. laidlawii and shown to be negative. The origins of virus strains, all of which had the physical properties and appearance typical of the MV-L2 group of viruses when viewed under the electron microscope, are given in Table 1. MV-L2 was supplied by Dr R. N. Gourlay, and cloned using indicators M/1305/68 and 0-305.

The medium used for growth of Acholeplasma strains was glucose-serum (GS) broth or agar (Gourlay & Leach, 1970), with 0-002 % calf thymus DNA. An agar base made up of 10 ml of PPLO agar (Difco) with penicillin 200 units/ml, thallium acetate 1:8000 and 0-002 % phenol red was poured into 90 mm Petri dishes and dried at 37 °C for 30 min. GS agar (40 ml) at 48 to 50 °C was added as the second layer, the third layer consisting of...
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3·0 ml of GS agar mixed with 0·3 ml of a 48 h GS broth culture of *A. laidlawii* indicator strain. In the latter part of the study, 0·0026 % tetrazolium chloride was added to the second and third layers as the contrast produced by its reduction to the red formazan facilitated plaque counting. After addition of virus, the tetrazolium containing layers were covered with 18 to 20 ml of PPLO agar to seal them off from the atmosphere.

Titres of virus preparations expressed as plaque forming units/ml (p.f.u./ml) were determined by a dropping method using phosphate buffered saline (Dulbecco solution A) with 0·2 % bovine serum albumin fraction V (PBS–BSA) as diluent. Portions (0·02 ml volumes) of virus dilution were dropped on to the surface of a triple layer plate seeded with the desired *A. laidlawii* indicator strain, and allowed to absorb into the agar at room temperature. Finally, the sealing layer of PPLO agar was added, allowed to set, and the plates incubated at 37 °C for 48 h before being examined for plaques.

Table 1. Origins of virus strains and cross resistance patterns

<table>
<thead>
<tr>
<th>Virus</th>
<th>Indicator strain for propagation and cloning</th>
<th>Plaquing ability on <em>A. laidlawii</em> strains 0-305, 0-216 and M/1305/68 resistant to:*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>V1</td>
</tr>
<tr>
<td>V1</td>
<td>0-298</td>
<td>+</td>
</tr>
<tr>
<td>V2</td>
<td>0-324</td>
<td>-</td>
</tr>
<tr>
<td>V4</td>
<td>0-324</td>
<td>+</td>
</tr>
<tr>
<td>V5</td>
<td>0-324</td>
<td>+</td>
</tr>
<tr>
<td>V7</td>
<td>0-318</td>
<td>+</td>
</tr>
<tr>
<td>MV-L2</td>
<td>M/1305/68</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, no reduction in plaquing ability; -, no plaques produced.
† See text.
‡ Reduced plaquing ability on V2 resistant clones of *A. laidlawii* 0-216.
§ Reduced plaquing ability on V5 resistant clones of *A. laidlawii* 0-305.

The virus isolates in the series V1 to V7 were found to differ in their host ranges, each showing a preference for the indicator strain in which it was isolated, propagated and cloned. The plaques produced all ranged in size from 0·5 to 2·0 mm with the exception of V5 which produced 'pin-point' plaques. On lawns of non-preferred indicator strains, the viruses failed to produce discrete plaques at high dilutions, or occasionally produced an area of indistinct clearing only at low dilutions. In some instances the production of plaques was erratic.

Rabbit antiserum to MV-L2 was obtained from Dr R. N. Gourlay who had prepared it against virus grown in strain M/1305/68. Virus neutralizing antibody titres were determined by a slight modification of the static method (Clyde, 1974). Equal amounts of appropriately diluted (5 × 10^4 p.f.u./ml) virus suspension and antiserum to MV-L2 were mixed, allowed to stand at room temperature for 1 h and drops from Pasteur pipettes of uniform bore applied to seeded layer plates.

The extent of virus activity, as shown by the degree of clearing of the indicator lawn was expressed on a scale of 0, ±, + or ++, the titre of the antiserum being the highest dilution at which some neutralizing activity occurred compared to the control.

The results of neutralization tests of the virus isolates as compared to MV-L2 are shown in Table 2. The neutralization titres for MV-L2, V1 and V4 are alike. V5 and V7 behave similarly, there being evidence of some neutralization but at a lower titre than for MV-L2. V5 failed to be neutralized even at the lowest dilution of antiserum tested.

To show adaptation of MV-L2 to *A. laidlawii* strain BN1, on which the initial titre was
lower than on M/1305/68, tenfold serial dilutions of the virus were applied to layer plates seeded with these two strains. Washes were taken of segments cut from the plates showing plaques at maximal virus dilutions on BNI and M/1305/68. These washes were titrated to show enhancement of titre on strain BNI as a result of passage in this strain. Similar studies were also carried out using V₂ with indicator strains o-216 and o-305.

A freeze-dried sample of V₁ was inoculated in GS broth together with *A. laidlawii* strain M/1305/68. Ten similar transfers were made. After the first and last passages, layer plates of the broth cultures were prepared, washes harvested, filtered through a 220 nm Millipore membrane, and titrated on layer plates seeded with various *A. laidlawii* indicator strains.

The initial titre of MV-L₂ on host strain BNI was almost 10000-fold lower than on host strain M/1305/68. After the first passage on BNI, the few plaques obtained titred out better on BNI than on M/1305/68.

**Table 2. Neutralization test results**

<table>
<thead>
<tr>
<th>Viruses</th>
<th>1/200</th>
<th>1/400</th>
<th>1/800</th>
<th>1/1600</th>
<th>1/3200</th>
<th>1/6400</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV-L₂</td>
<td>o</td>
<td>o</td>
<td>±</td>
<td>±</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>V₁ (few)</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>V₂</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V₃</td>
<td>o</td>
<td>(few)</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V₄</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V₅</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*±, ++, +++, Virus activity as visualized by clearing of the indicator lawns; few, a few plaques seen; o, no plaques seen.

A lyophilized V₂ sample, when initially titrated, produced clearing at a higher dilution on layer plates seeded with host strain o-216 than on o-305. Subsequently, washes of V₂ after first and second passages in strain o-305 showed no activity on *A. laidlawii* o-216 but continued to produce plaques in moderate numbers on o-305.

Similarly, V₁ showed adaptation to *A. laidlawii* M/1305/68 by producing a titre of $6 \times 10^6$ p.f.u./ml on this strain after ten passages in GS broth cultures of M/1305/68 as compared to an indistinct clearing at a dilution of $10^{-3}$ with the freeze dried material. Adaptation to M/1305/68 was associated with a reduction in titre by $10^3$ p.f.u./ml on strain o-216.

Reconstituted lyophilized virus was inoculated into GS broth seeded with the *A. laidlawii* strains from which resistant clones were desired. After 24 h incubation the broths were serially diluted, plated out, and the plates incubated for a further 24 to 48 h. Three to four of the colonies so produced were selected and tested for resistance to the virus. In some cases, this method was unsuccessful, and a selection method was used. Areas of clearing produced by virus were cut out of the indicator lawn and cultured in GS broth. These broth cultures were used to seed further indicator plates, which were exposed to virus, and the process repeated. After three exposures, cultures were serially diluted and colonies selected for resistance testing. Clones were considered resistant if high titre preparations of the virus used to prepare them failed to produce any visible clearing on indicator plates sown with them.

Cross resistance tests were performed by titration of viruses on lawns of the various resistant clones. Resistant clones of *A. laidlawii* strain o-305 were readily obtained to virus isolates V₂, V₄, V₅ and MVL-2; of strain o-216 to V₁ and V₂; and of strain M/1305/68 to MV-L₂.
This was evident by their failure to clear, or occasionally to clear only partially, when exposed to the virus they were made resistant to on triple-layer plate lawns. Also, the washes of these clones were shown to shed virus producing an area of clearing on lawns of indicator strains.

Stable resistant clones of *A. laidlawii* strains o-305 and o-216 to V<sub>7</sub> and of M/1305/68 to V<sub>1</sub> were difficult to produce. Whenever obtained, such resistant clones shed virus initially, and then failed to do so after subculture or storage. The cessation of virus shedding activity was associated with a return to susceptibility in these clones, which then behaved like the original indicator strains. Other V<sub>1</sub> resistant clones of M/1305/68 and V<sub>7</sub> resistant clones of o-305 and o-216, produced by the selective method did not shed viruses and remained resistant on subculture and storage.

Cross resistance patterns on clones prepared from *Acholeplasma* strains o-305, o-216 and M/1305/68 enable separation of the viruses into groups as shown in Table 1. V<sub>4</sub> showed an identical pattern to MV-L<sub>2</sub>, as did V<sub>5</sub> to V<sub>2</sub>. V<sub>1</sub> showed a reduced ability to plaque on V<sub>2</sub> resistant clones of *A. laidlawii* o-216, though plaquing of V<sub>2</sub> was not reduced on V<sub>1</sub> resistant clones of o-216. Some reduction in the plaquing ability of V<sub>4</sub> on strains of *A. laidlawii* o-305 resistant to V<sub>5</sub> was noted.

The MV-L<sub>1</sub> type of viruses have been extensively studied by Maniloff et al. (1974). By serology, u.v. inactivation kinetics, host range and one-step growth kinetics, they have concluded that MV-L<sub>1</sub> is a group of related but not identical viruses like many others, e.g. T-even bacteriophages. That heterogeneity amongst MV-L<sub>2</sub> may also exist has been indicated by these authors. The results of our investigations confirm this.

Host range studies indicate that the virus isolates vary in their ability to form plaques on different indicator strains of *A. laidlawii*. Different titres observed on the different indicator strains may be due to adaptations of the virus to the host strains on passage. Gourlay & Wyld (1972) have noted that plaque morphology and titre are difficult to control. Maniloff et al. (1974) have shown that MV-L<sub>2</sub> viruses are host modified and restricted. Our experience with MV-L<sub>2</sub> as well as with V<sub>1</sub> and V<sub>2</sub> viruses confirm these observations.

With the exception of V<sub>5</sub>, there were no significant differences in virus neutralization by specific anti-MV-L<sub>2</sub> antiserum. This indicates that all these viruses are of MV-L<sub>2</sub> type, possibly with some minor antigenic differences on the surface.

It is thought that infection with MV-L<sub>2</sub> virus, like MV-L<sub>1</sub>, is not invariably lytic, the infected cells continuing to grow while producing progeny virus. We believe that some component of the host cell membrane is retained in the virus envelope during the process of virus release, and that the presence of this component accounts in part for the heterogeneity of the MV-L<sub>2</sub> group of viruses as shown by our serum neutralization tests.

Two types of virus resistant clones of *A. laidlawii* were isolated to some of the viruses. In the first, carriage of virus was associated with resistance, and the loss of virus with a return to susceptibility. Thus, it seems likely that partially resistant mutants were selected. Once the virus is lost, introduction of further virus may result in a sufficient differential in growth rates between infected and uninfected *Acholeplasma* cells to allow plaque formation. The second type of resistant clone isolated by the selection method may represent a true mutation to resistance. Such clones were not shown to carry virus and did not readily revert.

The mechanism of this resistance and therefore, the basis of the observed heterogeneity of virus isolates V<sub>1</sub> to V<sub>7</sub> requires further study.

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Public Health Laboratory
Dulwich Hospital
London SE22 8QF

R. J. PHILLPOTTS
K. K. T. PATEL
D. G. ft. EDWARD

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