PROJECT REPORT No. 318

DEVELOPMENT OF FIELD TEST KITS FOR THE DETECTION OF BARLEY YELLOW MOSAIC VIRUS AND BARLEY MILD MOSAIC VIRUS

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DEVELOPMENT OF FIELD TEST KITS FOR THE DETECTION OF BARLEY YELLOW MOSAIC VIRUS AND BARLEY MILD MOSAIC VIRUS

by

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Abstract

Barley yellow mosaic virus (BaYMV) and *barley mild mosaic virus* (BaMMV) have been reported from all areas of the UK where winter barley is grown intensively. Yield loss in susceptible cultivars has been estimated at 30-50%. UK cultivars of barley have been identified which are resistant to the viruses and some are on the HGCA Recommended List. However, susceptible cultivars often give higher yields or have better quality and agronomic characteristics. Many crops are incorrectly diagnosed as suffering from minor or major trace element deficiencies when in fact the symptoms are due to barley mosaic virus. Considerable expense is incurred on agronomic inputs, attempting to solve problems which are actually due to virus infection. This expenditure would be avoided if rapid field diagnosis could be achieved. The aim of this project was to develop a field test kit for BaYMV and BaMMV, which would yield a reliable test result in 5-10 minutes.

Mice were immunised with purified BaYMV supplied by our scientific collaborators, IACR Rothamsted. Several fusions were carried out, but all antibodies raised to BaYMV were IgM. IgM antibodies are not suitable for use in lateral flow device development. When fresh infected plant material became available and virus was purified at Central Science Laboratory (CSL), subsequent immunisations and fusions resulted in IgG clones being produced, IgG being the preferred isotype for subsequent development. One of the IgG clones was selected as the antibody of choice, Y52, and was purified and tested by ELISA. Despite these monoclonals performing well in traditional ELISA, a suitable LFD could not be developed. The LFD's produced, when validated on the material supplied, were not sensitive enough when compared with ELISA or Taqman confirmation. No background was produced, i.e. no non-specific binding, which had been a problem with all IACR antibodies tested previously. The conclusion is that the BaYMV LFD could only be commercialised if further validation indicated that the tests are sensitive enough for field detection, on fresh material. During the last year no BaYMV samples had been received to CSL to further validate the test.

All the anti-BaMMV monoclonal antibodies supplied to us as tissue culture supernatant from IACR were IgM's, and all had an extremely low titre. These were unsuitable for either an ELISA test or LFD. Towards the end of the project, 2 further clones were obtained from a contact in France. These were purified and one is now used in an LFD. The LFD produced detects BaMMV specifically in leaf sample within 7-10 minutes. The sensitivity of the assay was at least 10 times less sensitive than a comparative ELISA, but adequate for detection in field samples.These LFD's

were sent out to various Arable Research Centre sites for field validation. One batch of devices was supplied to ARC and another batch to IACR. To date only the ACR results have been returned. The test results from the LFD's correlated well with the ELISA test on the same material.

Despite numerous events, including Cereals 2002, promoting the BaMMV LFD as well as making the test available to purchase as a Pocket Diagnnostic product, (see www.pocketdiagnostic.com), at a discounted rate for validation purposes, as well as making the test available to all the UK PHSI, no further validation on field samples has been achieved. It is believed that the BaMMV test performs adequately in the field on fresh material, but further validation is still required to build customer confidence. Unfortunately, during the last year the incidence of BaMMV and BaYMV has been low, and hence validation has not been possible. Last year only single samples were received and in that situation we advise that more sensitive methods for detection and confirmation purposes should be employed, namely ELISA and Taqman. Only when numerous samples are required at field locations would the LFD be a benefit to the user.

Summary

Overall project aim

The aim of this project was to develop a field test kit (or kits) for *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) which was also expected to yield a reliable test result in 5-10 minutes.

Barley yellow mosaic virus and *barley mild mosaic virus* LFD development

Antibody production

Our aim was to produce polyclonal and monoclonal antisera to BaYMV and incorporate them into an on-site field test in a lateral flow test format. Antibody development would also generate a reliable laboratory based ELISA test.

Specific objectives and milestones achieved

1. Collect infected plant material from the field (ARC). - by March 2000

Infected plant material was collected from the field during 2000, but we also collected it as and when it was possible to do so during the course of the project in order to get supplies for screening and purification.

2. ***Purify BaYMV from field material (IACR). - by June 2000****.*

Purifications of BaYMV were carried out at both IACR and CSL and used to produce polyclonals and monoclonals.

3. ***Purify expressed coat protein of BaYMV (CSL). - by Jan 2000****.*

This milestone was cancelled in discussion with HGCA - it was originally foreseen that expressed protein would be used to produce antibodies but in fact we used purified virus preparations instead.

4. Purify BaMMV from infected material (IACR). - by Feb 2000.

BaMMV was purified at IACR and used to screen BaMMV monoclonals (see below)

5. Screen polyclonals to BaYMV(IACR/CSL). - by October 2000.

Polyclonal anti-BaYMV sera was produced and worked well.

6. *Screen monoclonals to BaYMV (IACR/CSL) - by June 2001*.

Monoclonal anti-BaYMV antibodies were produced and screened. These worked well in the ELISA format. Purified fractions of these mponoclonals were incorporated into the LFD, with limited success. Background was eliminated but poor detection of the positive material supplied was obtained. Despite efforts to improve sensitivity this was not possible. However fresh material is generally higher titre, and hence the test may detect fresh field material, unfortunately no samples were received to confirm this.

7. *Screen monoclonals to BaMMV (IACR/CSL)- by June 2001*.

All BaMMV monoclonals produced by IACR were found to be IgMs and unsuitable for use – they were replaced by French monoclonals.

8. Test antibodies in lateral flow kits (CSL). - by December 2001.

All antibodies produced were tested in LFD formats.

9. Evaluate test kits in field (ARC). - by March 2002.

The BaMMV test kit was evaluated for use in the field. Results from this were promising however no more additional field samples were received from either IACR,ARC, PHSI or commercial growers, to enable further validation.

The BaYMV antibodies were not produced early enough to allow validation in LFDs during the original project life, and no further samples have been received.

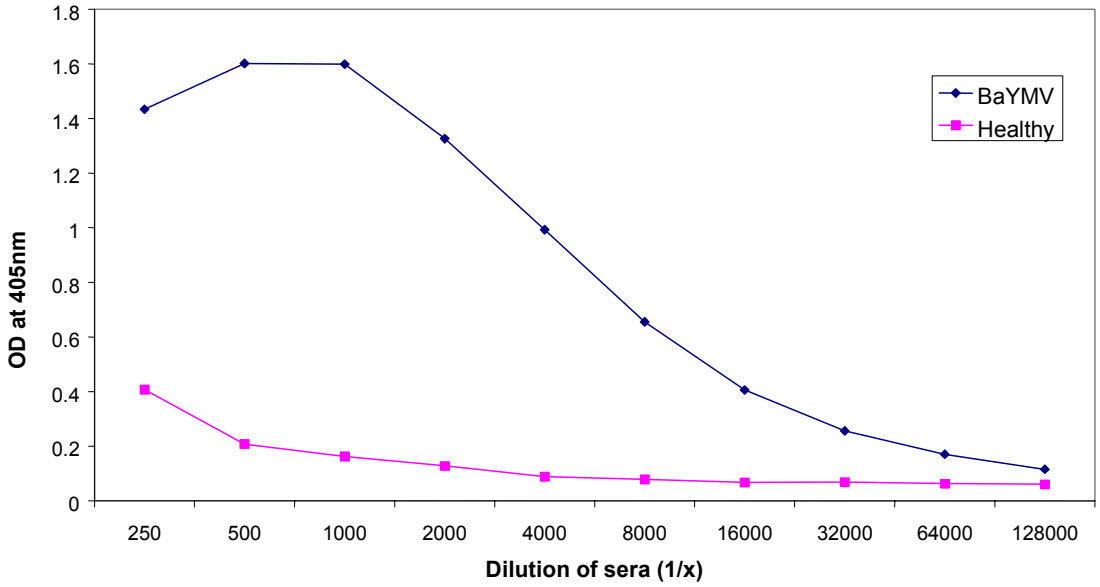
Purification of BaYMV

The virus was prepared by grinding/blending the material and centrifuging over a sucrose cushion. In summary, 1mM PMSF was added to the supernatant and centrifuged over a sucrose cushion. This was repeated 3 times. CsCl was added to the supernatant and the solution was centrifuged at 112,000g overnight. The virus forms a band '*in situ*' which was drawn off using a bent needle and syringe. This liquid was then centrifuged at 97,500g for 4 hours to pellet the virus. This method is described in further detail in the technical detail section.

Development of polyclonal anti-BaYMV antibodies

BaYMV was purified from infected plant material and 2 rabbits were immunised with these purified preparations of BaYMV at 100µg/ml in Freunds complete adjuvant. Three subsequent injections were then given at four weekly intervals in Freunds incomplete adjuvant and blood was taken for testing four weeks after the last injection. The first bloods were tested using a plate-trapped antigen ELISA format using anti-rabbit as the detecting antibody. Of all the polyclonal antibodies produced in house, R596 responded most strongly, and is now the antibody of choice for coating the plates. R596 also cross reacts with BaMMV.

Figure 1. Response of polyclonal anti-serum R596 to healthy and BaYMV infected barley material

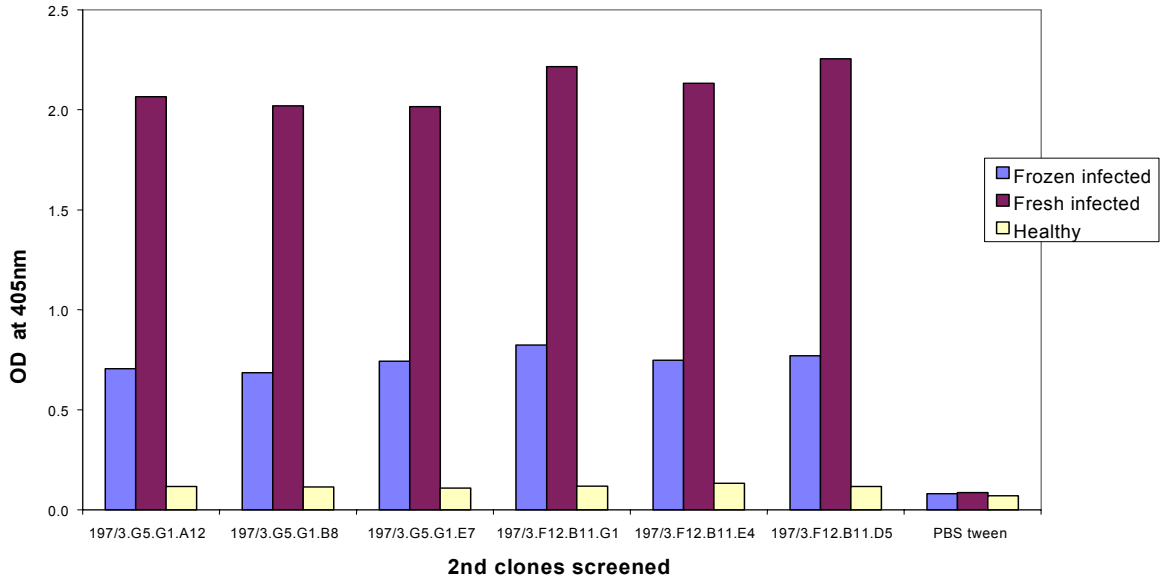


Development of monoclonal anti-BaYMV antibodies

BaYMV was purified from infected plant material (as described previously) and 6 mice were immunised, every 2 weeks, over a period of 6 weeks. Blood was taken at week 8 and screened in a triple antibody sandwich (TAS) ELISA for response. The selected spleen was fused with SP2/0 AG14 myeloma cells and the resulting hybrids screened. The selected hybrids were cloned twice by minimal dilution and screened in a TAS ELISA. This is described in further detail in the technical detail section later.

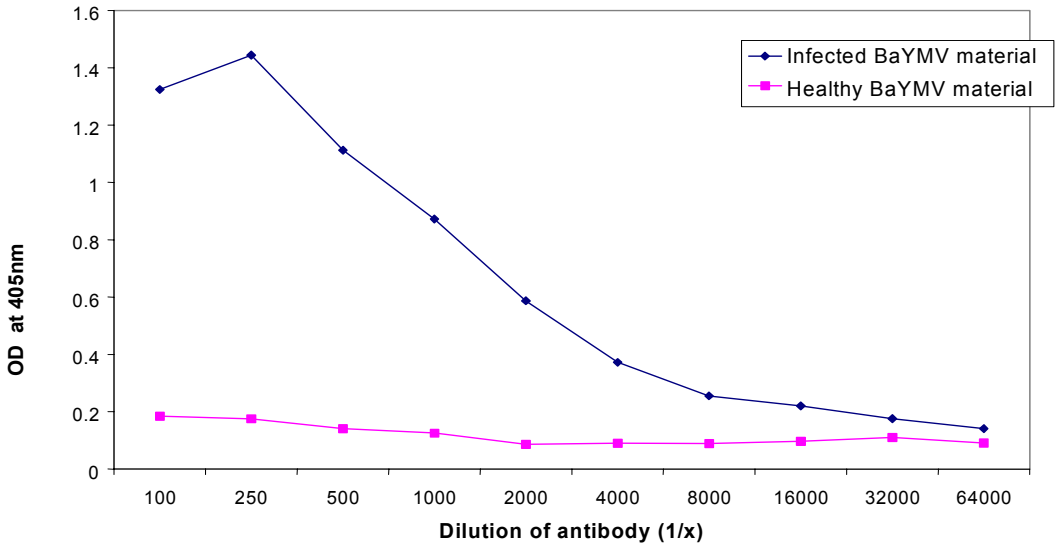
Figure 2 shows the results of screening 6 second clones using fresh virus infected field material.

Figure 2. Pre-purification screening results of 6 clones from fusion 197 using fresh and frozen infected BaYMV material.



These 6 clones were individually bulked up and purified on a Protein G column. All were isotyped as IgG. After further screening, one clone was selected for LFD development: clone 197/3.F12.B11.G1 was allocated the York number Y52.

Figure 3. Post-purification screening results of clone Y52 (197/3.F12.B11.G1)



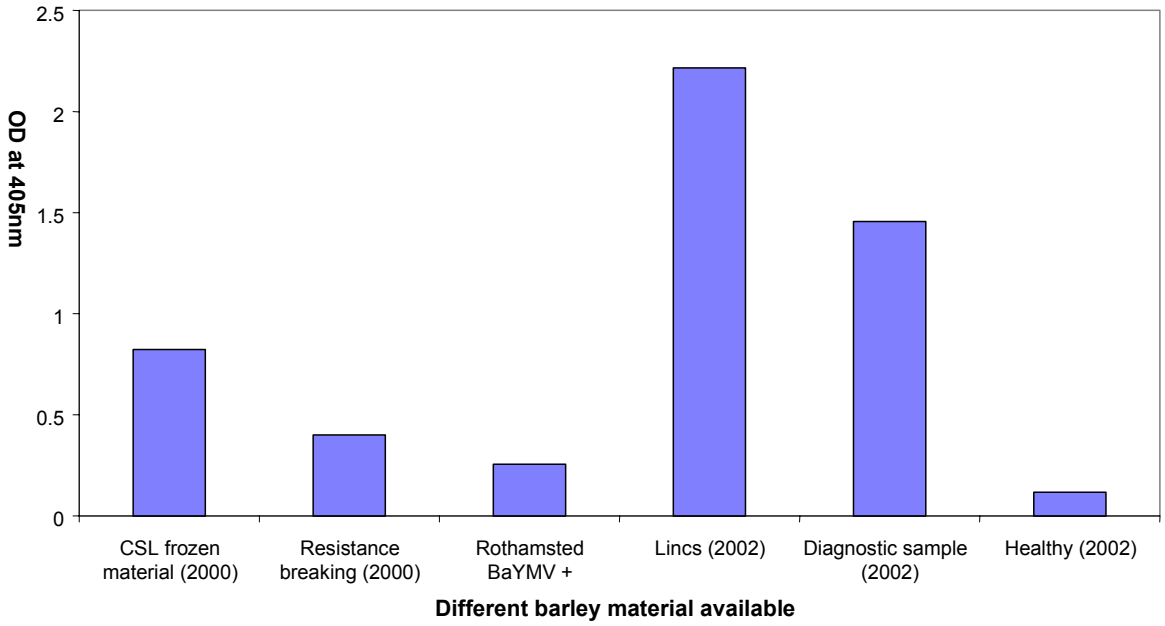
Material variations

A problem throughout this project was the availability of good quality infected material. As BaYMV is symptomatic for only a couple of weeks each year in early spring, it is essential that material is collected then. However in spring 2001 this was not posssible, due to the movement resrictions imposed during the foot and mouth disease outbreaks. This meant that all the initial screens were carried out using material that had been frozen for approximately 2 years. This material did not have a very high level of detectable infection, and was in limited supply so had to be used sparingly. Alternative frozen material was supplied by IACR, but this too had a low detectable level of infection.

It was not until February 2002 that fresh infected material became available to us from a site in Rothwell, Lincolnshire, courtesy of Paul Fenwick at Nickersons Seeds. This had a much higher level of detectable infection and allowed faster progress in screening.

Figure 4 below shows a comparison of the different material, when screened against Y52, mouse anti-BaYMV antibody.

Figure 4. Screening of all available barley material with clone Y52



A second problem encountered was the persistently high background on the healthy controls in the early stages of the project. Changing the buffer in which the plant material was ground, and eliminating polyvinyl pyrrollidone, caused a marked improvement in the background levels.

Development of monoclonal anti-BaMMV antibodies

All the anti-BaMMV monoclonal antibodies supplied to us as tissue culture supernatant from IACR were IgM's, and all had an extremely low titre. These were unsuitable for either an ELISA test or LFD. Towards the end of the project, 2 further clones were obtained from a contact in France. These were purified and used in LFD development.

Lateral flow device (LFD) development

Two independent lateral flow devices were developed, for BaYMV and BaMMV. The format selected was a double antibody sandwich and the general principles and criteria were employed as described by Danks & Barker (2001). All of the following antibodies were investigated for incorporation into the two LFDs:

3 different clones (anti-BaYMV) developed at CSL (Y52, 197/3.F12.B11.E4 and 197/3.F12.B11.D5) along with 2 other clones, EB7 and DD6 (anti-BaMMV) which were obtained from France from a contact of Christine Henry, and a range of IgM monoclonals and polyclonals available from IACR Rothamsted.

The antibodies were either sprayed at a range of dilutions in precise lines using the Biodot XYZ platform sprayer, or conjugated directly to latex particles. For each prototype, assays the following variables were looked at both independently and collectively:-

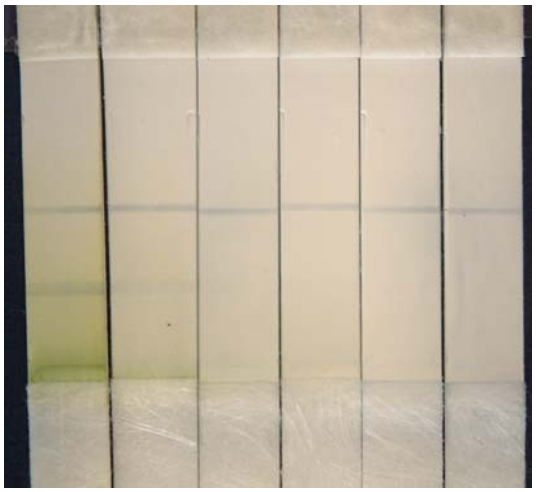
- antibody line application concentration
- membrane speed and type
- latex size, colour and reactive group
- buffer composition for blocking membranes
- extraction buffer and sample preparation

BaMMV LFD development

Figure 5 below shows two preliminary results for the BaMMV LFD development. The tests on the left are known as ‘wet assays’, this is the first stage of development and the assays are not held in plastic cassettes. The latex is mixed with the sample directly in a sample well, and not released from the conjugate pad. The ‘dry assays’, as shown on the right, are complete assays with the release pad included for latex deployment and the completed strip encased in a plastic housing. (a positive result, 2 lines, and a negative result, 1 line is indicated for both formats).

Figure 5

Each parameter was investigated and prototype devices were tested using a serial doubling dilution of infected sample, from 1/25 to 1/200 dilution and a healthy barley sample, see Figure 6 below. The two LFDs were compared for cross reactivity against other viruses and especially cross reactivity between BaYMV and BaMMV infected samples. The final criteria selected, and used to produce the final prototype and production models are commercially sensitive and hence not disclosed.



1/25 1/50 1/100 1/200 1/25 Healthy BaY

This shows the sensitivity of the BaMMV assay, with detection at 1/25 and 1/50 only and not 1/100 or 1/200 (lanes 1-4 respectively), but no detection of 1/25 BaYMV or healthy barley (lanes 5 and 6 respectively).

BaYMV LFD development

All available antibodies to BaYMV were used in combination in development of a BaYMV LFD. Two monoclonals were supplied by IACR Rothamsted, BaYMV 3 and 4, plus 2 clones from France, EB7 and DD6, and Y52. A further 2 polyclonal antibodies, 218 and 204 were also supplied by IACR Rothamsted, plus R596, which was developed at CSL. Unfortunately, no combination of any of these monoclonals or polyclonals produced a reliable LFD.

Development of plant sap extraction methods

A suitable extraction method was developed based on the 'Bottle and Ball' method previously developed by CSL. The modification and instructions include guidance on sample size and sampling regime, as described in the diagram below.

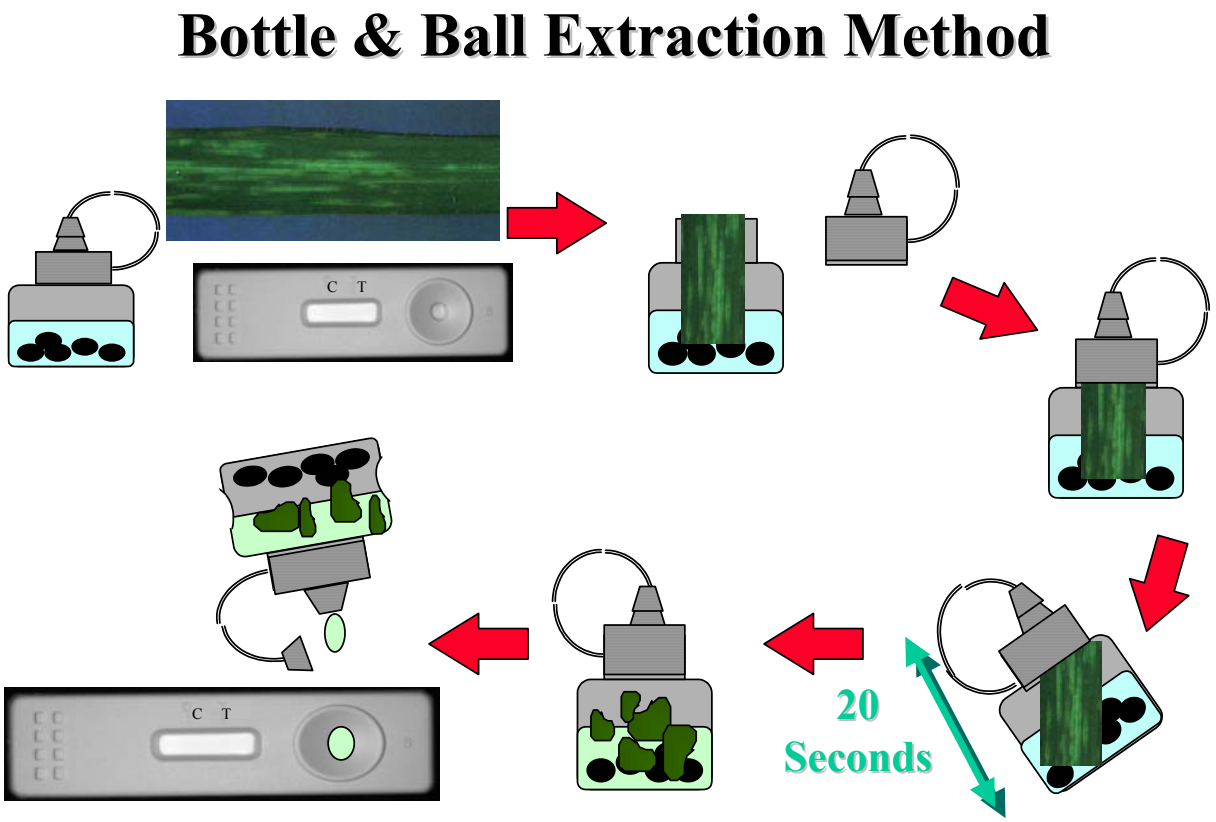


Figure 7: The "bottle & ball' extraction method modified for BaMMV.

Validation of LFD prototypes

Unfortunately due to delay in antibody availability only a small number of prototypes were made available. Samples have been provided to ARC and IACR Rothamsted. Of the two prototypes developed so far the BaMMV was validated in the field by ARC. No results were obtained from the samples proved to IACR Rothamsted.

The BaYMV test is not reliable at present, and has not yet been tested on field material. The BaMMV LFD has been distributed to several end-users including ARC and the DEFRA Plant Health Seed Inspectorate, for ongoing validation.

Sample	ID	LFD result	ELISA	Comment
1	7R1	+	1.01	
2	8R1	+	0.76	Weak LFD
3	8	-	0.64	Lowest ELISA
4	8	++	1.02	
5	9	+	0.85	Weak LFD
6	9	+	0.72	Weak LFD
7	9R1	-	0.54	Lowest ELISA
8	2S	+	0.93	Weak LFD
9	2S	+	1.04	Weak LFD
10	2S	++	1.138	
11	2S	+	0.73	Weak LFD
12	2SR2	+	1.208	
13	26	+	0.83	
14	26	+	0.89	
15	27	++	1.12	
16	27	+	0.85	

Table 1 Comparison of the LFD result carried out in the laboratory and the same sample tested by routine ELISA. Samples received from ARC.

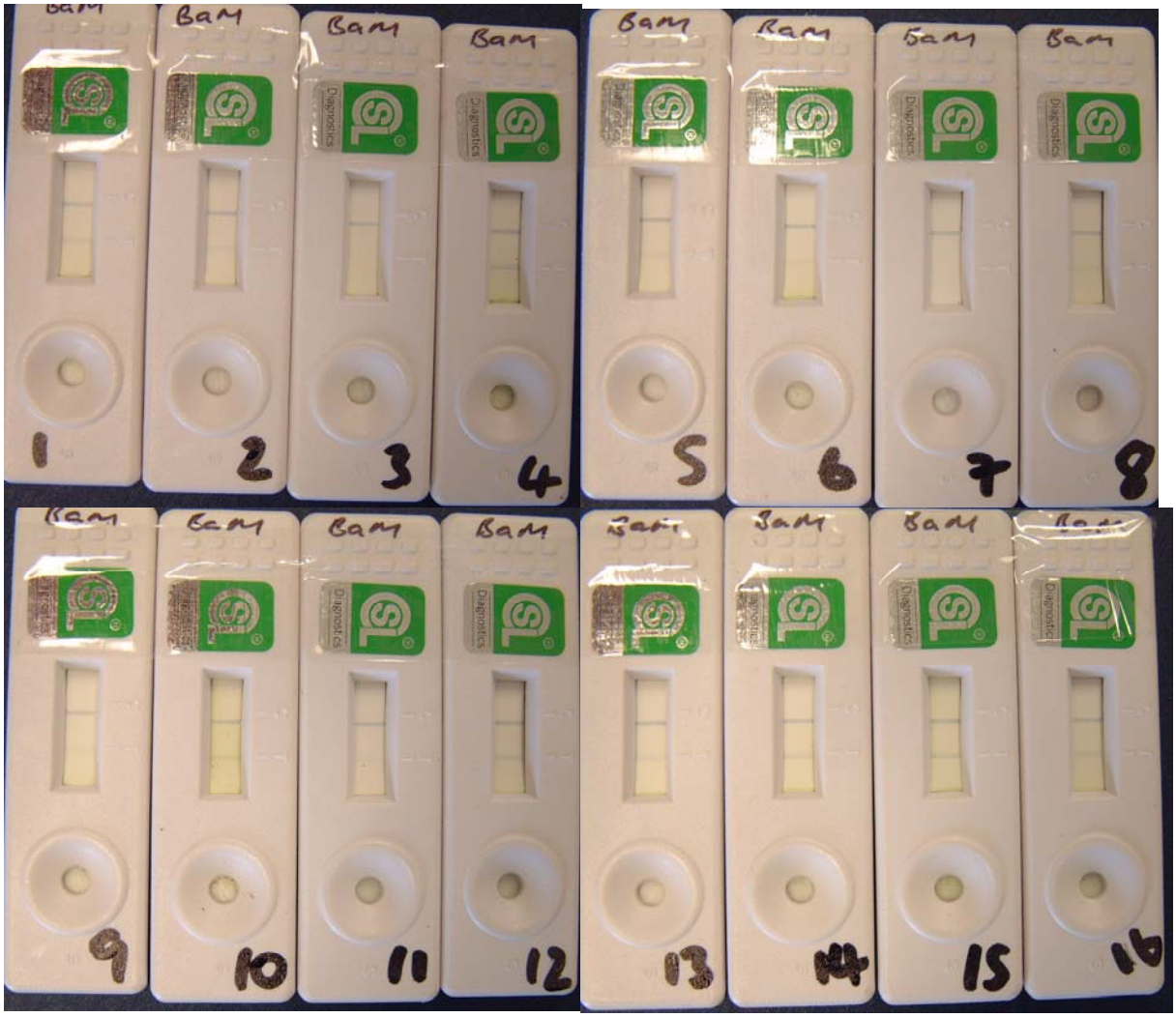


Figure 8 are the actual LFD results, showing the range of very clear positives (samples 1,4,10,12 - 16) and the two negative results (3 and 7), and weak LFDs (2,3,5,6,8,9,11).

The results so far showed the BaMMV assay to be reasonably effective in the field, when identical samples are compared to laboratory ELISA testing. The comments from the field were favourable. The BaMMV test is slightly slower than other plant pathogen kits developed by Pocket Diagnostics, but this is unavoidable due to the low titre of the barley viruses in the sample. The results do not indicate any false positives, but on some occasions false negatives were observed compared to ELISA data, but these two samples were identified as the lowest ELISA readings recorded. This is explained by the fact that the LFD is generally less sensitive that laboratory ELISA analysis, but could also be due to distribution of virus in the plant, hence sampling differences. Recent developments with real-time PCR (Taqman) (Mumford et al 2002) resulted in laboratory methods

far more sensitive than traditional ELISA. It is unfair to compare this method with the LFD in terms of sensitivity, although valid to use this approach in further confirmation of samples, particularly when mixed infections can occur, where one virus appears to dominate and suppress the second. By LFD or ELISA this would appear as a single infection, but analysis by Taqman has been used to subsequently indicate a mixed infection. This is clearly important when selection of cultivars is addressed.

Analysis of validation samples – 2003

Paul Fenwick (Nickersons Seeds) collected 2 samples in early 2003. These were suspected positive for both BaYMV and BaMMV, and were tested for both by ELISA, and for BaMMV only by LFD.

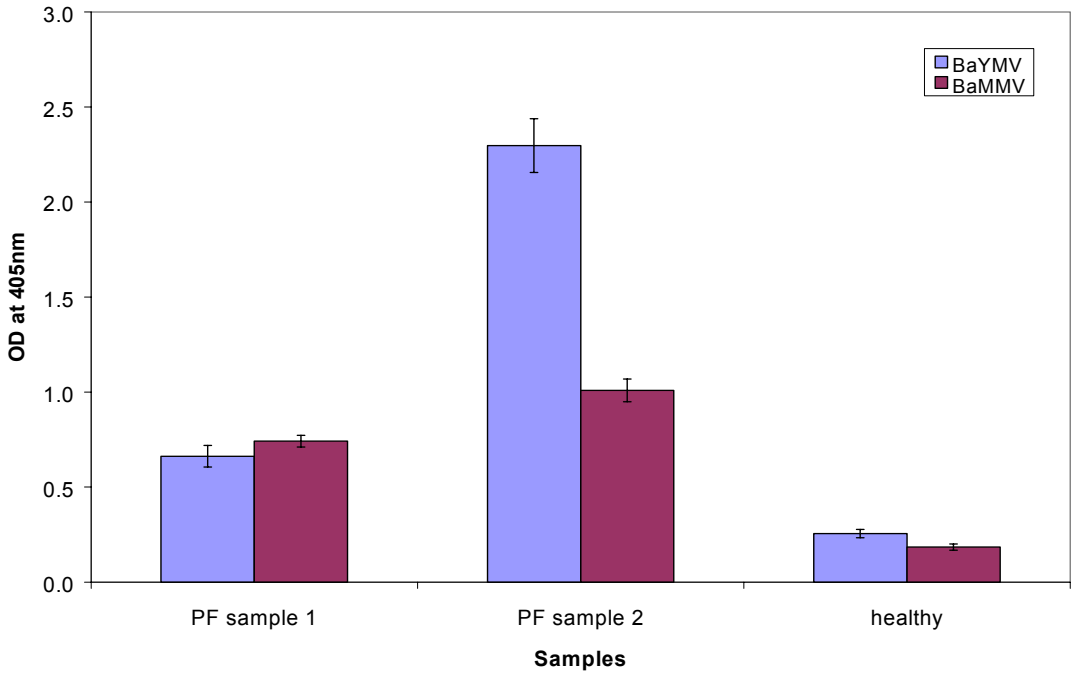


Figure 9. ELISA data of validation sample testing.

Figure 10. LFD testing of validation samples

The ELISA and LFD results for BaMMV testing agreed, both were positive. The ELISA test for BaYMV gave both a strong and a weak positive. Both samples were quite degraded when received through the mail, which could have affected the results.

Technical detail : Methods

Purification protocol for Barley yellow mosaic virus & Barley mild mosaic virus

1. Blend 100 g leaf pieces (fresh or previously powdered in liquid N_2 and stored at -70^{o}C) in 400 ml cold buffer A + 25 ml/l Triton X-100 + 1mM PMSF. Strain through muslin.

2. Low speed spin at 11,000 rpm (19,615g) for 15 min at 4^{o}C. Retain supernatant.

3. Centrifuge over a sucrose cushion: 6 tubes of 66 ml prep layered over 10 ml of 30% (w/w) sucrose in buffer A + 1mM PMSF.

4. Spin at 35,000 rpm (112,000g) for 2 hours at 4^{o}C.

5. Discard supernatant, drain and wipe excess liquid from tubes. Smear pellet and resuspend in 20 ml cold buffer A + 1mM PMSF. Allow to resuspend for a minimum of 1 hour at 4^{o}C.

6. Low speed spin at 5,500 rpm (3,507g) for 10 min at 4^{o}C. Retain supernatant.

7. Make up supernatants to 66 ml with buffer A + 1mM PMSF and centrifuge over a sucrose cushion: 6 tubes each of 66 ml prep layered over 10 ml of 30% (w/w) sucrose in buffer A + 1mM PMSF.

8. Spin at 35,000 rpm (142,000g) for 2 hours at 4^{o}C.

9. Discard supernatant, drain and wipe excess liquid from tubes. Smear pellet and resuspend in 8 ml cold buffer A + 1mM PMSF. Allow to resuspend for a minimum of 1 hour at 4^{o}C.

10. Low speed spin at 5,500 rpm (3,507g), 10 min at 4^{o}C. Retain supernatant and make up to 66 ml with buffer A + 1mM PMSF.

11. Mix supernatant with CsCl (5.15 ml prep + 2.85 ml stock 60% (w/w) CsCl solution per 13 ml tube)

12. Spin overnight (16-17 hours) at 35,000 rpm (112,000g) at 4^{o}C to band virus. Gradient is formed *in situ*.

13. Withdraw virus band using a bent needle and syringe.

14. Dilute 10-20 fold in buffer A and spin out virus at 29,000 rpm (97,500g) for 4 hours at 4^{o}C.

Buffer A: 0.1M Tri-potassium citrate (32.442 g/l)
0.01M EDTA di sodium salt (3.725 g/l)
pH adjusted to 7.0 with KOH if necessary

PMSF: Phenylmethylsulfonylfluoride: prepare as 50mM stock in isopropanol (8.7 g/l). Add to buffer solutions etc immediately before use. **PMSF is highly toxic**.

Immunisation schedule

At day 0, the mice are inoculated with 0.1ml (100ug/ml) antigen in complete Freunds adjuvant, subcutaneously. At weeks 2,4 and 6 the mice are inoculated intra peritoneally with 0.1ml antigen in incomplete Freunds adjuvant. At week 8, all mice are tail bled and the sera screened in a direct plate trapped antigen ELISA. The selected mouse is given a final boost of 0.1ml in PBS and sacrificed 4 days later. The spleen is harvested.

Fusion

The splenocytes are teased out of the spleen, washed twice in Hanks balanced salts solution, and counted. SP2/0 myeloma cells are counted. The ratio of spleen cells to myeloma cells used in a fusion is 3:1. Add the correct number of splenocytes and myeloma cells and centrifuge to remove any media. Keeping the cells at 37^{o}C, add 1ml of PEG over 1 minute , followed by 6ml of RPMI over 3 minutes, mixing continuously. Make up to 50ml with RPMI and centifuge at 2000rpm for 5 minutes. Discard the media, and resuspend in RPMI, 20% FCS, 1% HAT, 0.5% HES. 10ml is needed for each 96 well plate to be set up. Aliquot into 96 well plates, 100ul per well, and incubate at 37^{o}C 5% CO_2 for 14 days. Feed on days 3, 6 and 10.

Hybridoma screening and antibody development

All wells are screened at approximately day 14 by ELISA. Any selected ones are transferred into a 24 well plate and retested. Selected hybridomas are cloned by minimal dilution and screened, then cloned a second and final time. The final antibodies chosen are bulked up and 500ml tissue culture supernatant collected. If it is an IgG isotype it is purified on a protein G column.

The purified antibody is validated by ELISA and tested for suitability for inclusion in an LFD.

BaYMV ELISA protocol

1. Coat ELISA plate (Nunc maxisorp) with R596, 1:500 in coating buffer, 100ul per well
2. Incubate at 33^{o}C for 2 hours
3. Wash x3 with PBS / 0.02% tween
4. Grind 1g of plant material in 10ml grinding buffer
5. Add 100ul of plant sap per well

6. Incubate at 4^{o}C overnight
7. Wash x3 with PBS / 0.02% tween
8. Add Y52, diluted 1:1000 in PBS / 0.02% tween / 0.2% BSA, 100ul per well
9. Incubate at 33^{o}C for 1 hour
10. Wash x3 with PBS / 0.02% tween
11. Add rabbit anti-mouse IgG – alkaline phosphatase conjugated, 1:4000 in PBS/0.02% tween/0.2% BSA, 100ul per well
12. Incubate at 33^{o}C for 1 hour
13. Wash x3 with PBS / 0.02% tween
14. Add pNPP, 1mg/ml in substrate buffer, 100ul per well
15. Incubate at room temperature for 1 hour
16. Read at 405nm on plate reader

Buffers

Coating Buffer : 15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6

Grinding Buffer : PBS / 0.02% tween / 0.2% BSA

Substrate Buffer : 10% diethanolamine, 0.02% $MgCl_2$, pH 9.8

References

BLOCKLEY,A; METCALFE,E. ; WALSH, K.; BOONHAM,N. and MUMFORD,R.(2001). The reliable detection of Barley yellow and mild mosaic viruses using TaqMan. Poster presented at IXth Conference on Virus Diseases of Gramineae in Europe 21-23 May 2001 CSL, York, UK.

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