

A new approach to identifying La France disease

- microbiological tools

A report prepared for the

**New Zealand Vegetable and Potato
Growers Federation Inc.**

and the

**New Zealand Commercial
Mushroom Growers Federation**

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1 EXECUTIVE SUMMARY

This project set out to establish the reverse transcriptase PCR (RT-PCR) method for the detection of La France disease in New Zealand mushroom caps. It was supported by the New Zealand Commercial Mushroom Growers Federation and the New Zealand Vegetable and Potato Growers Federation.

RT-PCR detection of La France disease has been established in Australia as the routine method of detection and New Zealand growers wished to see the same technology introduced into New Zealand.

We obtained the protocol and primers needed for this method from Australia and tested it under New Zealand conditions. We also made an additional positive control. The method was then verified against mushroom tissue known to be infected with La France from Australia and the USA.

As a result of this study we have confirmed that the RT-PCR method is suitable for New Zealand conditions and that results are consistent and reliable. The conclusion from this work is that New Zealand mushroom growers should adopt this method because it is quicker, more sensitive and cheaper than existing methods.

This method offers opportunities to detect very early infections. Additional work could allow La France to be detected from mushroom compost and mycelium.

2 INTRODUCTION

Mushroom virus (La France disease) is a serious and potentially destructive disease. There is no treatment for it once it becomes established in a crop. Good farm hygiene and shed management, combined with the use of virus-free spawn are currently the most common approaches for avoiding this problem. Positive identification of La France disease is critical to the development of appropriate management strategies because many other conditions superficially resemble the symptoms of La France. Misdiagnosis can result in unnecessary costs to the grower and unsuccessful treatment. Early and correct diagnosis can alert the grower to the existence of the problem and enable a disease management strategy to be launched.

Two methods have been used to date to identify La France. Immunosorbent electronmicroscopy (ISEM) has been used to detect viruses in mushroom crops while the direct extraction and visualisation of double-stranded viral RNA (dsRNA) particles is well established. However, both methods, while acceptable, have short comings. ISEM tests are not specific for La France virus and the dsRNA test requires relatively large volumes of mushroom tissue.

Following the development of Polymerase Chain Reaction technology (PCR) methods, a reverse transcriptase PCR (RT-PCR) test has been established that allows La France to be detected in the early stages of mushroom development (Harmsen 1990). The test is also faster and more specific than all previous tests - some 5000 times more sensitive than the dsRNA test (Moran et al. 1995). A routine testing system using the RT-PCR method has been designed with funding from the Australian Mushroom Growers' Association and Horticultural Research Development Corporation. The system enables Australian mushroom growers to monitor their farms for the presence of La France.

The project described in this report was undertaken to explore the potential for transferring these RT-PCR methods to New Zealand and to establish them as the standard method for monitoring La France in this country.

3 METHOD

Based on Moran's results, the M1 primer was selected as the standard primer set for the detection of La France disease (Table 1).

Table 1: Primer set M1, relationship to dsRNA molecules, and predicted product size.

Primer pairs	dsRNA band	Product size (bp)
Upstream: TCA CAA GTG CGC CTA CTT TAG TGG C	M1	467
Downstream: TAG GCT TAA CGC GAA GCT TCA ACC G		

The M1 primer detects the dsRNA virus band M1 normally found in an infected mushroom (Fig. 1).

3.1 The PCR reaction

Mushroom extracts, prepared as described for dsRNA analysis, were used as templates for the RT-PCR reaction. RNasin (Promega), Superscript reverse transcriptase (BRL), and Taq DNA polymerase (Boehringer) were used according to the manufacturers' specifications for cDNA synthesis and PCR amplification. dsRNA was denatured for four minutes at 95°C in a DNA Thermal Cycler (Corbett).

The following cycling scheme was used:

- one minute at 94°C,
- one minute at 50°C,
- one minute at 72°C, for two cycles,
- 30 seconds at 94°C,
- 30 seconds at 50°C,
- one minute at 72°C, for 28 cycles,

- 30 seconds at 94°C,
- 30 seconds at 50°C,
- five minutes at 72°C, for one cycle.

Amplification products were analysed by electrophoresis in 1% agarose gels.

3.2 Rapid extraction of dsRNA sample

Rapid sample extraction was also tested. From five mushroom caps a 1 mm square of tissue was squashed in 30 µl of 100 mM Tris-HCl buffer containing 1M KCl and 10 mM EDTA, boiled for 10 minutes and then placed on ice. The sample was centrifuged for five minutes. A 1 µl aliquot of this extract was diluted in 9 µl of distilled water. From this dilute sample 1 µl was taken for the PCR reaction.

3.3 Reference samples

Mushroom caps with confirmed La France infection were obtained from Dr Moran, Victoria, Australia. She obtained these samples from Victorian mushroom farms where La France had been confirmed. The samples were then retested before being sent to New Zealand. Dr Romaine of Penn State University, USA, also supplied a freeze-dried sample of infected mushroom. This material was tested by Dr Romaine before being sent to New Zealand.

Both the Australian and American samples were tested by us using the dsRNA test and found to be heavily infected with La France. (Fig. 2). As a comparison, uninfected samples were drawn from the mushrooms sent in by New Zealand growers for routine examination using dsRNA methods. In addition, a reference positive control was cloned in the laboratory from a positive PCR reaction using the M1 primers. This positive control was needed to check that the PCR reaction had behaved normally.

3.4 Positive control cloning

1. 30 µl of the M1 product was extracted with equal volumes of chloroform and iso-amyl alcohol. Fractions were separated by spinning for 10 minutes at room temperature.

2. The top fraction was collected and precipitated with 0.2 volumes of ammonium acetate and an equal volume of isopropanol. The sample was spun for 10 minutes and the pellet was washed in 70% ethanol and redissolved in 20 μ l H₂O.
3. 1 μ l of this sample was ligated with the PGEMT vector and the ligated product was transformed into DH5 α and plated out on ampicillin plates. Twelve potential clones were selected and the plasmid extracted from them. All 12 plasmid preparations were run out on a 1% gel and examined for the presence of the correct-sized insert (Fig. 3).

Clone 1 was selected and aliquots of the plasmid preparation were mixed with glycerol and stored at -80°C for future use.

4 RESULTS

During the reaction large amounts of background product were produced. Examination of the Moran protocol showed that the high primer concentration (20 μM) may have been the cause. A test with decreasing primer concentration identified that the optimum primer concentration was 10 μM . Using 5 μM was also suboptimal (Fig. 4).

The M1 primer sets routinely amplified dsRNA from the infected reference material. The cloned positive control was also routinely amplified.

We did not detect any positives in the locally-produced mushrooms (Fig. 5).

The rapid extraction method was reliable and consistent.

5 DISCUSSION AND CONCLUSION

The RT-PCR method of detecting La France disease is a significant improvement on existing methods. It is quicker, more sensitive and cheaper than existing dsRNA methods. The inclusion of both positive and negative controls is required to ensure the validity of the results.

It is recommended that the New Zealand Commercial Mushroom Growers Association adopts this method as its standard diagnostic method for detecting La France disease.

6 REFERENCES

Harmsen, M.C. 1990: La France disease of the cultivated mushroom (*Agaricus bisporus*). Thesis. University of Groningen, The Netherlands. 83 p.

Moran, J.; Giles, R.; Irvine, G.; Reville, P. 1995: Improved mushroom virus diagnosis. Final report for HRDC project MU 300. 20 p.

7 FIGURES

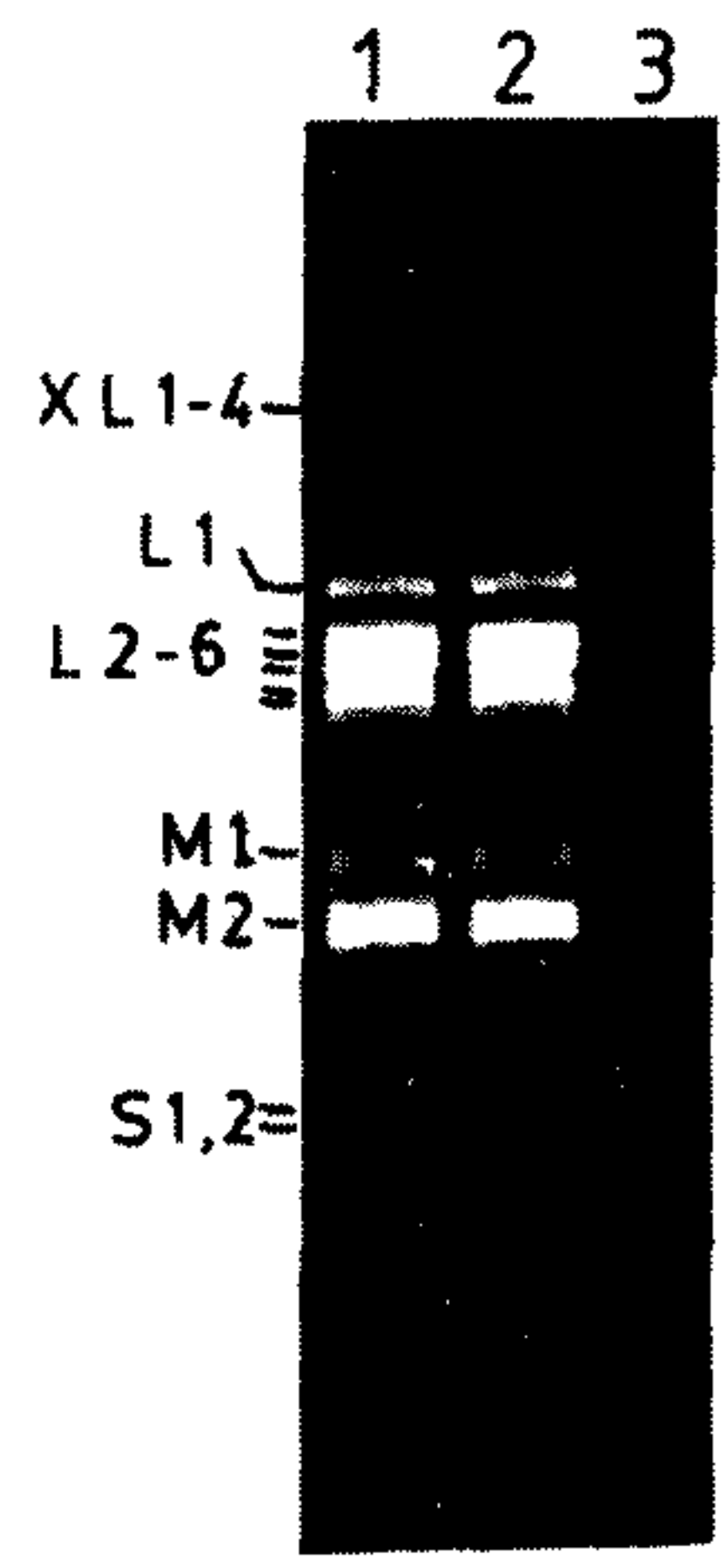


Figure 1: Typical dsRNA patterns showing the number and position of bands present in infected mushroom samples (from Harmsen (1989)).

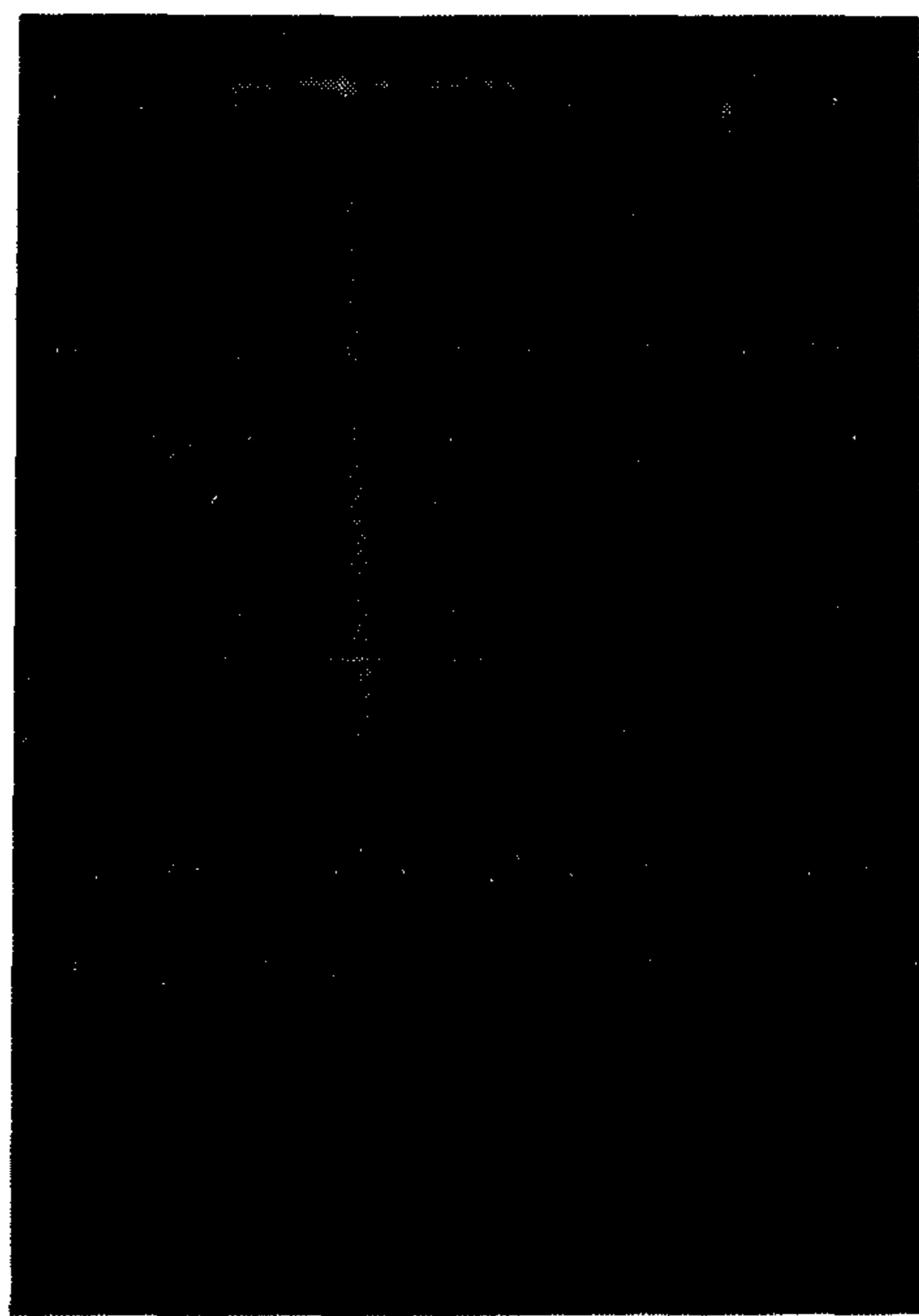


Figure 2: Typical dsRNA patterns isolated from infected mushroom tissue obtained from the USA and Australia.

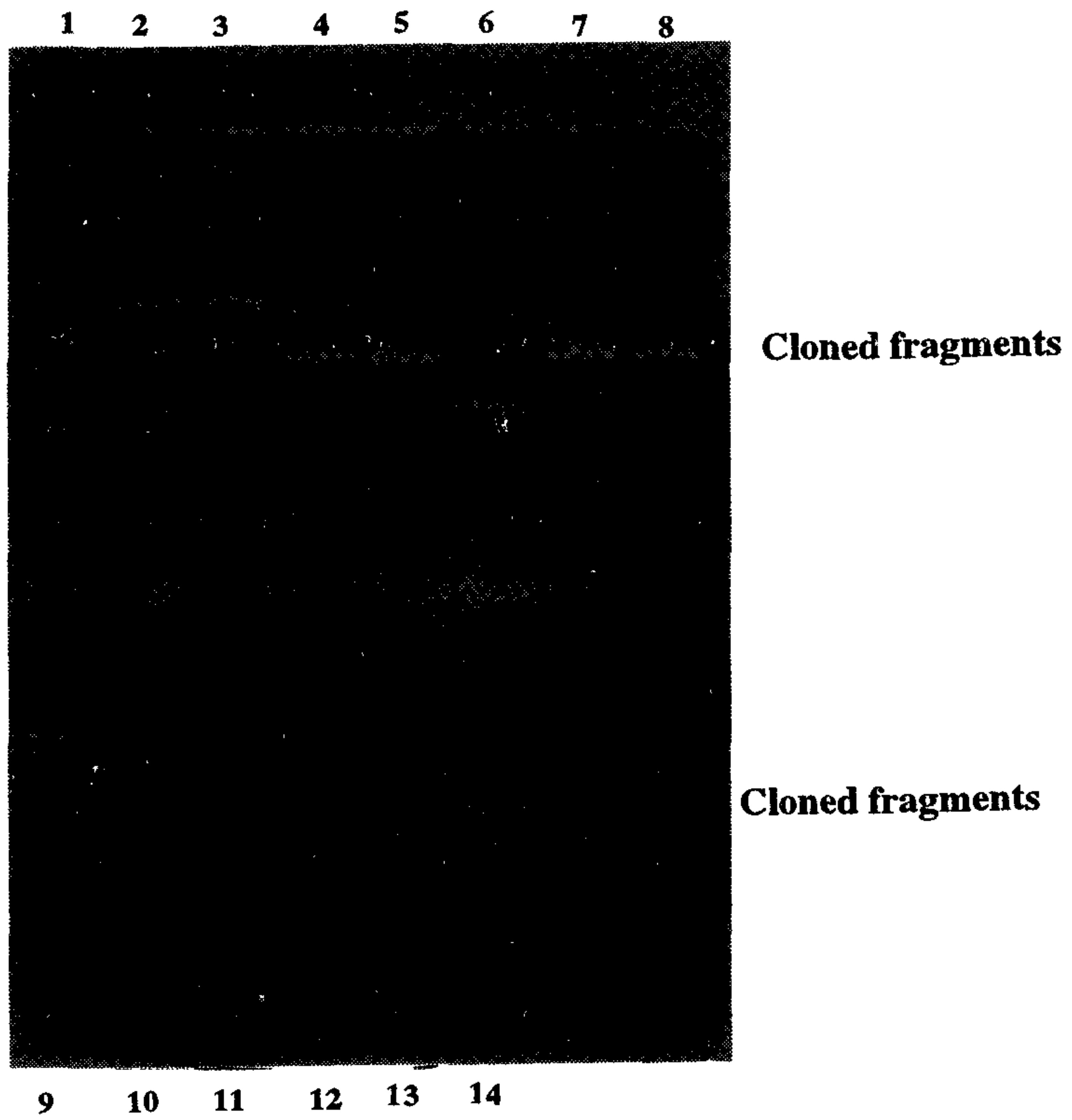


Figure 3: Cloning of M1 positive control. PCR-amplified plasmid DNA containing the M1 insert. Lanes 4, 5, 7, 8, 11 and 14 contain the M1 insert.

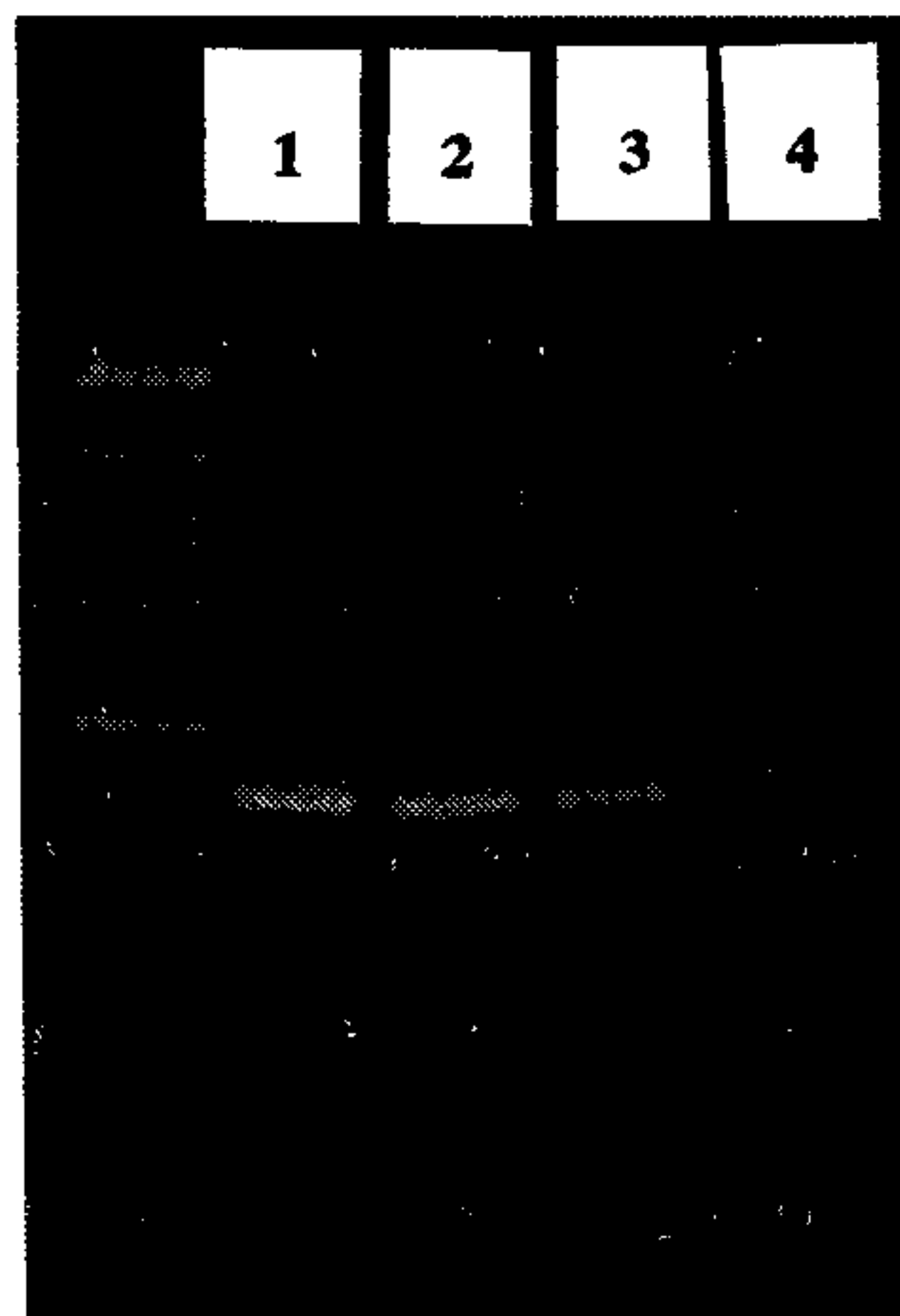
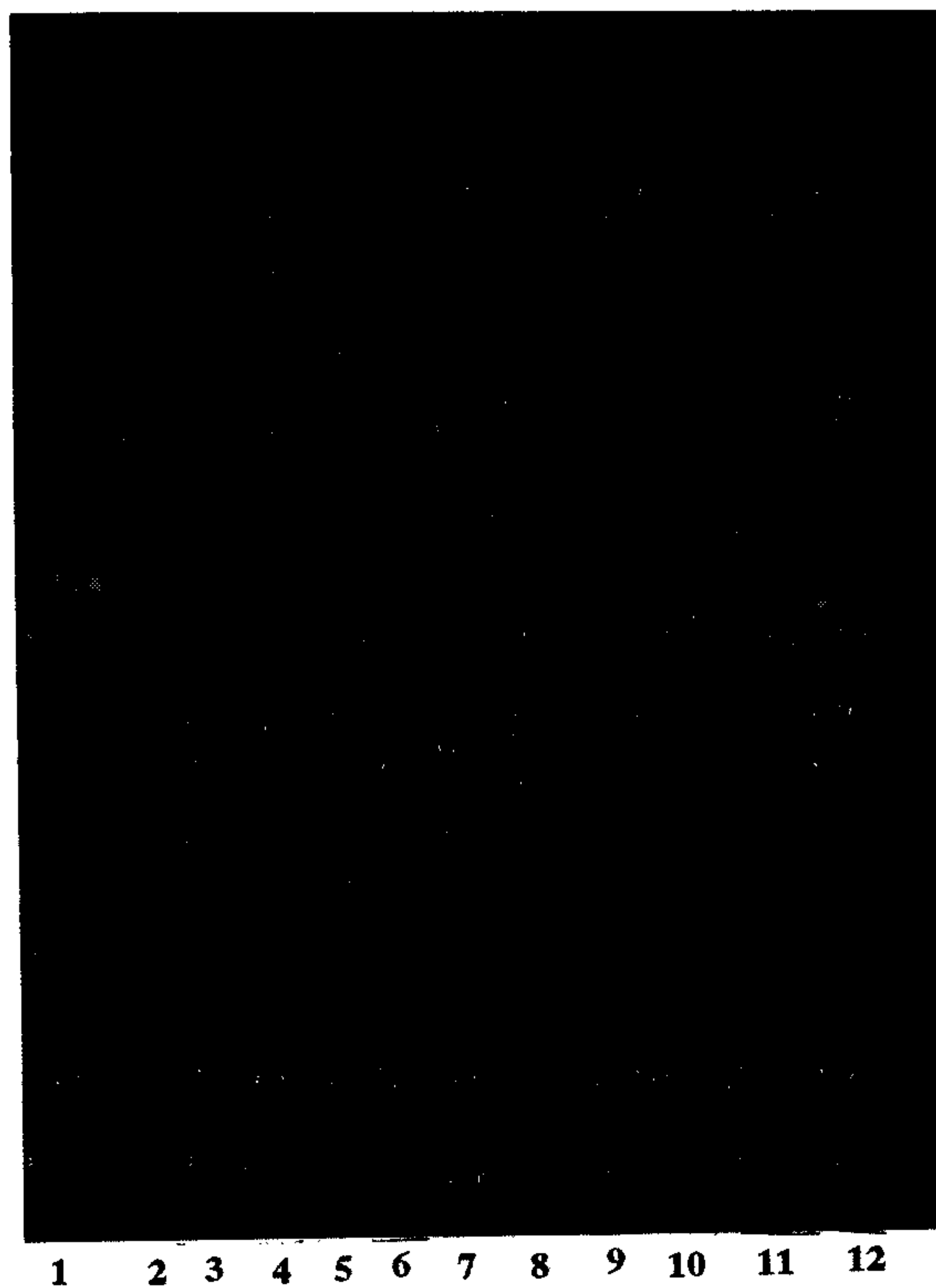


Figure 4: Effect of primer concentration on quantity of M1 amplified product. Lane 1 - molecular weight standard, Lane 2 - 20 μ l, Lane 3 - 10 μ l, Lane 4 - 5 μ l of both primers.



- | | |
|-------------------------|------------------------------|
| 1. 100 base pair ladder | 5-9 <i>New Zealand farms</i> |
| 2. Penn State, +ve RT | 5. L W, Rm 24, -ve |
| 3. -ve control | 6. L W, Rm 26, -ve |
| 4. Victoria, +ve | 7. M M, Sample I, -ve |
| | 8. M M, Sample II, -ve |
| | 9. M S, Crop 20, -ve |
| | 10. -ve control |
| | 11. +ve La France |
| | 12. 100 base pair ladder |

Figure 5: Amplification of M1 band from infected control samples, cloned positive control and uninfected mushroom samples from New Zealand farms.