ESTIMATING AMBER DISEASE IN GRASS GRUB POPULATIONS BY VISUAL ASSESSMENT AND DNA COLONY BLOT ANALYSIS

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ABSTRACT

Estimating levels of amber disease, caused by strains of the bacteria *Serratia entomophila* and *S. proteamaculans*, in populations of *Costelytra zealandica* is important for management of the pest. Since discovery of the disease, visual assessment has been the main method for disease estimation, but development of an amber disease specific DNA probe provides the opportunity to use bacterial colony blot analysis for detection of disease. Visual assessment and colony blot analysis of third instar grass grub populations produced similar estimates of disease and were highly correlated. The probe could also be used to determine the presence of disease causing bacteria in prepupal/pupal populations when visual disease symptoms were not evident.

Keywords: Grass grub, *Costelytra zealandica*, amber disease, *Serratia* spp., diagnostics, DNA probe.

INTRODUCTION

Amber disease is a bacterial disease of grass grub (*Costelytra zealandica* (White)) caused by strains of the bacteria *Serratia entomophila* and *S. proteamaculans* bearing a specific disease encoding plasmid, pADAP (Glare *et al.* 1993). The disease is recognised as a regulatory factor of grass grub populations (Jackson 1984; Jackson 1993) and strains of *S. entomophila* have been developed as the biological control product Invade (Jackson *et al.* 1992). Natural epizootics of amber disease are common among grass grub populations and sometimes occur at levels of up to 80% of larvae infected (Trought *et al.* 1982). Amber disease is a chronic disease of grass grub larvae and is characterised by cessation of feeding and gut clearance in the early stages of disease (Jackson *et al.* 1993). However, in mature larvae it is often hard to differentiate between diseased and healthy larvae as accumulated fat obscures the gut. Also, some larvae display temporary amber symptoms unrelated to infection by *Serratia* spp. Thus, it is important to develop objective indicators of disease which can be used in prediction of grass grub population change. DNA technology provides such an alternative. A DNA probe has been developed which is specific to the disease causing plasmid (Glare *et al.* submitted). In this paper we compare bacterial colony blot analysis using the DNA probe with visual diagnosis for estimating the level of disease in grass grub populations.

METHODS

Grass grub larvae were field collected from pastures in Canterbury where the grass grub biological control product Invade had been applied. From each site, the larvae were categorised as diseased or healthy on the basis of visual symptoms of disease (Jackson *et al.* 1993). Each larva was then cut into two in the region of the anterior thoracic segments and gut fluid was streaked in a short band across a plate of caprylate thallous agar (CTA) (Starr *et al.* 1976) and incubated for 3 days at 30°C. A plasmid-bearing and plasmid-free strain of *S. entomophila* were included on each CTA plate as the positive and negative controls respectively. The resultant bacterial colonies were then transferred onto Hybond-N membrane (Amersham, Life Science) as per the manufacturer's

instructions. DNA was fixed to the membrane by UV cross-linking. Development of the probe, an *Eco*R1 segment of pADAP designated pMS20 is described elsewhere (Glare *et al.* submitted). 32 P-DNA probes were prepared by random *in vitro* labelling following the procedure of the *rediprime* DNA labelling system (Amersham, Life Science) and purified by passage through a sephadex G-50 column (Sambrook *et al.* 1989). All prehybridisation, hybridisation and washing steps were carried out in a Biometra (OV3) oven at 65°C. Colony blots were prehybridised in 2xSSC (0.3 M NaCl, 30 mM sodium citrate) for 15 mins, followed by prehybridisation buffer (1 mM EDTA, 0.5 M Na_2PO_4 , 7 % SDS) for 1-3 hrs. Twenty microlitres of denatured probe (100°C, 5 mins) were then added and the blots hybridised overnight. After three 15 minute washes in 2xSSC, the blots were exposed to x-ray film (Kodak, X-OMAT AR) overnight. Following developing, the autoradiographs were visually scored using the positive and negative controls as references.

In a further test, late instar larvae and pupae were taken from four untreated grass grub populations in Canterbury and processed as outlined above. In addition to the gut smear, 10 μ l of whole insect macerate, produced by blending with 1 ml sterile deionised water with a Ultra-turrax T25 blender at 15000 revs for 7 seconds, was inoculated onto the CTA medium.

Results were analysed using chi-squared test for independence and the McNemer Change test corrected for continuity (Siegel and Castellan 1988).

RESULTS

Both methods produced a similar overall estimate of disease (Table 1). Of the 409 larvae examined, visual examination categorised 35% of larvae as diseased, while the use of the specific DNA probe produced 37% positive bacterial colonies. In each of the samples the correlation between methods was highly significant ($P < 0.001$) using the chi-square test, although there were a considerable number of samples which produced contradictory test results. For example, Sample 1 produced a significantly greater ($P < 0.01$ McNemer test), number of colonies that probed positive among apparently healthy larvae than would be expected while Sample 3 produced a greater number ($P < 0.01$) of colonies probing negative among visually diseased larvae than expected.

TABLE 1: Amber disease assessment from grass grub larvae using visual assessment and colony blot analysis of bacterial colonies isolated from larvae.

Sample	Collection time	Diseased visual assessment		Healthy visual assessment		Chi square test χ^2	McNemer Change Test χ^2
		Probe+	Probe-	Probe+	Probe-		
1	July 1995	45	5	19	31	31.6***	7.0**
2	May 1996	33	10	16	83	51.4***	1.0 ns
3	June 1996	23	13	2	70	19.5***	6.6**
4	June 1996	11	3	3	42	26.7***	0.2 ns
Total larvae		112	31	40	226		
% in each category		27	8	10	55		

Grass grub collected from the October 1996 sampling were mostly in the pupal and prepupal stage with a small number of retarded larvae showing symptoms of disease. Probing, however, revealed that 17-50% of insects were carrying the disease causing bacteria with greater numbers of bacterial colonies with homology to the probe detected from the macerates than the smears (Table 2).

TABLE 2: Detection of amber disease causing bacteria among prepupal and pupal grass grub populations sampled in October 1996 (P, pupae; L,

larvae) by visual assessment and DNA probe of isolated bacterial colonies.

Site	n	Visual disease (A)	Colony probe+ smears (B)	Colony probe+ macerates (C)
Murdoch	17 (15P,2L)	1	6	9
Willis	10 (6P,4L)	0	5	5
Porter	20 (19P,1L)	0	4	4
WIRS	12 (5P,7L)	3	2	4
Total	59	4	17	22
McNemer χ^2			A vs B = 9.6**	B vs C = 1.5 ns

DISCUSSION

Amber disease symptoms, gut clearance and cessation of feeding, are clearly associated with infection by disease-causing strains of *Serratia* spp. (Stucki *et al.* 1984; Jackson *et al.* 1993) but accurate visual assessment of the level of disease in a population is difficult. In the tests described in this paper, both visual analysis and DNA dot blot methods produced similar estimates of disease in feeding larvae but each method had its strengths and weaknesses. Visual assessment has been used most frequently to date and is rapid and relatively low cost. The weakness of visual assessment is that amber symptoms are occasionally produced from other causes (which could be the case with Sample 3), and the estimate of disease will vary depending on the experience of the observer. Isolation of bacteria from individual larvae for confirmation of disease is possible, but agar plate based identification is a laborious process (Stucki *et al.* 1984; O'Callaghan and Jackson 1993a). It is also impossible to tell, from the visual symptoms of disease, whether larvae are infected once they have reached the fatty stage of the mid third instar. The DNA probe pMS20, on the other hand, has homology to a fragment of the disease causing plasmid, pADAP and hence will only detect disease causing bacteria. In combination with the *Serratia* spp. selective medium, CTA, the probe gives consistent results but it is more time consuming and hence more expensive than visual examination. If, however, there are high numbers of non pathogenic bacteria in the insects these can outgrow the pathogenic strain on the CTA agar and the latter will remain undetected. This is an alternative explanation for the discrepancy between larvae showing disease symptoms and positive probes in Sample 3.

The greatest advantage of the DNA probe is that it allows detection of disease causing bacteria in insect stages where disease symptoms are not apparent. This may have been the reason for the high number of positive probed colonies from apparently healthy larvae in Sample 1. In the October sample, disease was only apparent among the few remaining larvae, but probing revealed that 20 - 50% of grass grubs were carrying the bacteria. The effects of bacteria on pupae are not known, but adult beetles have been implicated as important factors in the transport and distribution of pathogenic strains of bacteria (O'Callaghan and Jackson 1993b).

In summary, both methods of estimation of amber disease have strengths and weaknesses. Visual assessment is adequate where a quick result is required for disease estimates among early third instar larvae. The DNA probe is more appropriate for confirmation of ambiguous visual results, for assessments at the pupal and adult stages and also as a technique which can be used for detection of bacteria in all life stages for experimental studies to determine ecology of bacteria and epizootiology of disease.

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