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A RAPID, SENSITIVE, USER-FRIENDLY METHOD FOR DETECTING STORAGE MITES

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A RAPID, SENSITIVE, USER-FRIENDLY METHOD FOR DETECTING STORAGE MITES

by

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Abstract

The study reported here forms the first part of work to develop a rapid method for detecting storage mite pest infestation in cereals and their derived products. This was achieved by examining the suitability of an existing monoclonal antibody for detection of mites by immunoassay. The specificity of the antibody was investigated to 13 species of mite and four species of insect pest. Its sensitivity was examined by studying the reaction to different numbers of mites.

The results demonstrated that, in the absence of grain, the monoclonal antibody could:

- detect and accurately quantify unknown numbers of the cosmopolitan food mite, *Lepidoglyphus destructor*, ranging between 0-100 adult mites;
- detect both dead and live adult mites of *L. destructor* and its faeces;
- detect the house mite, *Glycyphagus domesticus* with similar sensitivity to *L. destructor*.

In the presence of grain the sensitivity of the assay decreased. This was probably because the existing antibody had not been raised for this purpose. However, further investigation using an additional, polyclonal, antibody gave satisfactory results in accurately detecting a range of 0 to 100 mites seeded in 5g of both wheat and barley. This demonstration of an immunoassay for the quantification of *L. destructor* mites in stored grain is a significant advance on previous immunoassays which have focused on detecting mites in dust.

The work reported here has proven the potential of the immunoassay method for the rapid and sensitive detection of storage mites. It is clear that the existing monoclonal antibody is insufficient by itself for detecting mites in cereal grain and, although we have shown how this can be overcome by using the additional polyclonal antibody, this would still have limitations. The supply of polyclonal antibody is not guaranteed and the assay will only detect the Glycyphagid mites *L. destructor* and *G. domesticus*.

It is therefore proposed that future work should raise an antibody against a common storage mite protein rather than crude extracts of mites, with the additional precaution that the selection pressure includes lack of reactivity to cereal grains. This would allow detection of a wider range of mite pests even in the presence of grain. Previous experience in related studies suggests that this approach would be highly successful and the best way to exploit the encouraging results obtained in the present study.

Summary

Storage mites threaten the quality of UK grain, oilseeds and their derivatives. They damage stored grain and cause occupational health problems to farmers and workers in the grain and milling industry. Storage mites have also been found in processed cereals destined for human consumption and cited as the cause of potentially life-threatening anaphylaxis after ingestion of foods with heavy infestations. Pesticide resistance is now widespread in all three major mite species found infesting grain in farm and central grain stores, *Acarus siro* (L.), *Lepidoglyphus destructor* (Schrank) and *Tyrophagus longior.*

Grain quality is difficult to control and prove to potential purchasers if it cannot be monitored. Several methods have been suggested to determine the presence of mites but all have disadvantages being for example time consuming or unreliable. The purpose of the present study was to investigate the use of an existing antibody as the basis for an immunoassay method of detection which would overcome these disadvantages.

The existing antibody, monoclonal 42B6, was found to be very sensitive and able to detect single mites for both Glycyphagid species *L. destructor* and *Glycyphagus domesticus.*

The antibody responded best to these two species and to the less common *Aleuroglyphus ovatus.* Protein analysis by Western Blot showed there was a common band at 39 KDa in all three of these species, which would explain their reactivity with this antibody. Response was marginal with *Tyrophagus longior, Tyrophagus putrescentiae, Acarus siro, Acarus farris* and *Blomia tropicalis.* There was virtually no reactivity with *Tyrophagus palmarum, Glycyphagus ornatus, Caloglyphus berlesei* or the house dust mite, *Dermatophagoides pteronyssinus.* Neither was there any significant reactivity with any of four storage insect pest species tested, *Ahasverus advena, Oryzaephilus surinamensis, Sitophilus granarius* and *Cryptolestes ferrugineus.*

Of the different life stages of *L. destructor*, adults had the greatest response while responses to tritonymphs and nymphs were marginal and there was no response with eggs. Live adult mites of this species, dead adult mites and mite faeces all gave strong responses. Responses to spent food and to a mixture of faeces and food were marginal, while there was little response to food alone. Detecting dead mites and mite faeces could be of an advantage for even if live mites are removed from a sample, mite allergens in the product could still be hazardous to health.

Although the antibody was able to detect mites alone, it could not do so in the presence of either barley or wheat. It is possible that this was because the antibody, which is known to work by recognising a sugar moiety, was at least partly bound to cereal carbohydrates and therefore unavailable to detect the glycoprotein antigen. The problem was overcome by using an additional, polyclonal, antibody in an extra amplification step. This demonstration of an immunoassay for the quantification of *L. destructor* mites in stored grain is a significant advance on previous immunoassays which have focused on detecting mites in dust.

The work reported here has proven the potential of the immunoassay method for the rapid and sensitive detection of storage mites. It is clear that the existing antibody is not sufficient by itself for detecting mites in cereal grain and, although we have shown how this can be overcome by using an additional polyclonal antibody, it would not be without its own limitations. The supply of polyclonal antibody is not guaranteed and the assay is limited to the detection of the Glycyphagid mites *L. destructor* and *G. domesticus.*

It is therefore proposed that future work should raise an antibody against a common storage mite protein rather than crude extracts of mites, with the additional precaution that the selection pressure includes lack of reactivity to cereal grains. This would allow detection of a wider range of mite pests even in the presence of grain. Previous experience in related studies suggests that this approach would be highly successful and the best way to exploit the encouraging results obtained in the present study.

INTRODUCTION

Storage mites threaten the quality of UK grain, oilseeds and their derivatives. They damage stored grain directly (Boczek, 1991), make it unpalatable to livestock and reduce its suitability for milling (Wilkin and Stables, 1985; Wilkin and Thind, 1984). Storage mites cause occupational health problems to farmers and workers in the grain and milling industry (Cuthbert *et al.*, 1979; van Hage-Hamsten *et al.*, 1985; Blainey *et al.*, 1989). They have also been implicated in the transmission of fungi, bacteria and prions (Steinbrink and Boer, 1984; Domenichini *et al.*, 1992; Wisniewski *et al.*, 1996). Mites have also been found in processed cereals destined for human consumption (Anon, 1996a, Thind and Clarke, 1999) and been cited as the cause of potentially life-threatening anaphylaxis after ingestion of foods with heavy infestations (Matsumoto *et al.*, 1996).

Modern agriculture is driven by customer needs so markets often exist only if strict quality standards are met. For example, European Community regulation 689/2 states in Article 2 that, amongst other things, cereals offered for intervention 'must be free from live pests (including mites) at every stage of their development' (Anon, 1996b).

To avoid problems from mites, stored grain management in the UK relies heavily on bulk admixture with pesticides. This extensive practice has resulted in the development of pesticide resistance in all three major mite species found infesting grain in farm and central grain stores, *Acarus siro* (L.), *Lepidoglyphus destructor* (Schrank) and *Tyrophagus longior* (Gervais)(Prickett, 1997; Thind and Muggleton, 1998).

Grain quality is difficult to control and prove to potential purchasers if it cannot be monitored. There is a growing need for measurement technology that matches the scales and speeds of the operations of harvesting, storage and processing. Several methods have been suggested to determine the presence of mites. These include sieving (Wilkin, 1982), flotation analysis (Thind and Wallace, 1984), measurement of guanine content (Bischoff *et al.*, 1989) and use of baited mite traps (Thind, 1997). However, sieving is unreliable (Lynch and Thind, 1985), while flotation analysis is time-consuming and requires knowledge of mite morphology to distinguish between different species. Guanine determination suffers from lack of specificity as substances other than mites can contribute to the amount obtained (Hallas *et al.*, 1993) and mite traps are user-friendly but require several days to be effective.

Immunoassays are based on the detection of specific antigens by antibodies. Potentially, they are reliable, rapid and do not require expert knowledge. Specific assays for house-dust mites with immunochemical methods such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) have been proposed for the house dust mite, *Dermatophagoides pteronyssinus* (Trouessart)(Luczynska *et al.*, 1989) but there has been less investigation of such methods for storage mites. Recently, Härfast *et al.* (1996) used a monoclonal antibody (mAb), 42B6, in an inhibition ELISA to detect and quantify storage mite allergens from *L. destructor* in dust samples collected from barns. However, it was not known if their assay could be used to detect mites in grain or other commodities. The aim of the work reported here was to establish whether this antibody could form the basis of a method for the early detection of storage mites in samples of unmilled cereals.

METHODS

Mites

The mite species used in this experiment were: *L. destructor,* G6 strain; *A. siro,* 9258/2; *Glycyphagus domesticus* (DeGeer), GAG; *T. longior,* T96; *Tyrophagus putrescentiae* (Schrank), T13; *Tyrophagus palmarum* (Oudemans), T32; *Acarus farris* (Oudemans), A17; *Blomia tropicalis* (Bronswijk), BL1; *Caloglyphus berlesei* (Michael), C3; *Aleuroglyphus ovatus* (Troupeau), AL2; *Dermatophagoides pteronyssinus* (Troussart), (D3); *Glycyphagus ornatus* (Kramer), Berlin; *Cheyletus* sp., field strain.

Cultures of mite strains were reared in the dark at 20° C and 80% RH in 600ml cell growth flasks (115 mm long × 165 mm wide × 50 mm high), on a finely ground, sterilized and conditioned mite diet consisting of flour and dried yeast (1:3 w/w). The different life stages were separated by using 500, 185, 150 and 75μm aperture sieves. Spent food (food that is left in an expired culture) and faeces, were separated from the mites by 20, 150 and 425μm aperture sieves.

Insects

Insect species tested were *Ahasverus advena* (Waltl), 7&8 wk; *Oryzaephilus surinamensis* (L.), 7&8 wk; *Sitophilus granarius* (L.), Windsor; *and Cryptolestes ferrugineus* (Stephens), C124s.

Insect strains were reared in the dark at 25° C and 70% RH in 0.75litre Kilner jars. *A. advena and O. surinamensis* were fed a diet of wheatfeed, rolled wheat and yeast (5:5:1 w/w). *S. granarius* was fed on whole wheat only.

Antigen release

Homogenisation of mites/insects was performed by the use of plastic homogenizers (Biomedix). Mites/insects were crushed in 50 μl of phosphate buffered saline (PBS) for 30 sec. The protein concentration of the extract was then determined (Biorad). Solutions were diluted to the appropriate protein concentration in PBS and used as antigen in ELISA's.

Antigen release from mites in grain

Five gram samples of wheat and barley were infested with known numbers of mites plus 1 ml PBS. The samples were placed in plastic bags (12cm × 12.5cm) containing a gauze mesh inner (9.5cm × 12cm) to retain the grain from the antigen supernatant - this aids the removal of the antigen when pipetting. Preliminary samples were homogenised by the use of a stomacher for 1 min. However it was shown that it was preferable to use a mechanised ball-bearing device. In this latter method a roller fitted with ball bearings is used to crush the mites in the grain for 1 min while the bags are trapped in position on a flat surface. This method was used for all the quoted results

Antibodies

The monoclonal antibody cell line, 42B6, was donated from the Karolinska Hospital, Sweden. Hybridomas were produced according to standard procedures (Köhler and Milstein, 1975).

Although tissue culture supernatant from cultures of cells secreting antibodies is of sufficient concentration for certain studies, more concentrated preparations are necessary for the development of diagnostic and quantification assays. For this reason a proportion of antibody was purified from tissue culture medium according to standard methods using Protein G (Pharmacia, Uppsala, Sweden).

A small aliquot of monospecific polyclonal antibody was also donated from Withington Hospital, Manchester.

General information on ELISA

Numerous variations of the ELISA have been developed but in this study only three were applied. All use a colour reaction to quantify an antigen of interest. The intensity of the colour reaction depends on the amount of specific enzyme present, the colour reaction occurring when a specific substrate is added. The amount of enzyme present is determined by the amount of antigen present because the enzyme is attached as a label either to the antibody which recognises the antigen or to a second antibody which recognises that first antibody. Thus:-

INDIRECT ELISA:

> Antigen bound directly to ELISA plate, amount present determined by antibody with enzme label attached.

DOUBLE ANTIBODY SANDWICH (DAS):

> Antibody which recognises antigen bound directly to ELISA plate and used to 'trap' antigen from solution. Amount of antigen trapped determined by second antibody with enzyme label.

TRIPLE ANTIBODY SANDWICH (TAS):

> Antibody which recognises antigen bound directly to ELISA plate and used to 'trap' antigen from solution. Amount of antigen trapped determined by second antibody. Amount of second antibody present determined by third antibody with enzyme label attached.

All three of these ELISA techniques were performed (Harlow and Lane, 1988) using Nunc MaxiSorp microtitre plates. Negative controls consisted of wells coated with PBS or extracts made from grain containing no mites. The optical density was measured photometrically at 405nm in a microtitre plate reader after 30 min incubation

Indirect ELISA

Microtitre plates were plates coated overnight at 4°C with 50 µl of mite homogenates. After incubation, excess antigen was removed from the wells and the plates were rinsed in three changes of 0.1M PBST (PBS plus 0.05% v/v Tween 20, Sigma), for 3 min each time. Plates were incubated for 30 min on a plate shaker at room temperature with 100 µl per well of PBST plus 5% Marvel milk powder. Plates were rinsed as

before and incubated with either tissue culture supernatant (indirect ELISA's)([1:5] diluted in PBST) or purified mAb (DAS and TAS ELISA's)([1:1000] diluted in PBST) for 1 hr on a plate shaker at room temperature. After three more rinses, 50 µl of second antibody conjugate (alkaline phosphatase conjugated rabbit anti-mouse IgG, Sigma) at 1:1000 dilution in PBST was added to the wells and the plates were shaken for 45 min at room temperature. Finally after three more rinses with PBST, 100 µl of alkaline phosphatase substrate (KPL) was added to each well and incubated at room temperature in the dark.

DAS ELISA

Microtitre plates were coated with 100 µl purified mAb [1:1000] diluted in PBS and left to incubate at 4°C overnight. After washing, the wells were incubated with 200 µl of blocking buffer (PBST plus 5% of Marvel milk powder) for 30 min at room temperature on a plate shaker. After washing, the wells were incubated with 100 µl of mite homogenate or grain extract for 1 hr at room temperature on a plate shaker. After further washing, the wells were incubated with 100 µl of alkaline phosphatase conjugated mAb (Linkit), [1:100] diluted in PBST, for 1 hr at room temperature on a plate shaker. Finally after three more rinses with PBST, 200 µl of alkaline phosphatase substrate (KPL) was added to each well and incubated at room temperature in the dark.

TAS ELISA

Microtitre plates were coated with 100 µl purified pAb [1:2000] diluted in PBS and left to incubate at 4°C overnight. After washing, the wells were incubated with 200 µl of blocking buffer (PBST plus 5% of Marvel milk powder) for 30 min at room temperature on a plate shaker. After washing, the wells were incubated with 100 µl of mite homogenate or grain extract for 1 hr at room temperature on a plate shaker. After washing, the wells were incubated with 100 µl of mAb [1:1000] diluted in PBST, for 1 hr at room temperature on a plate shaker. After washing, the wells were then incubated with 100 µl of second antibody conjugate (alkaline phosphatase conjugated rabbit anti-mouse IgG, Sigma) [1:1000] diluted in PBST, and the plates shaken for 45 min at room temperature. Finally after three more rinses with PBST, 200 µl of alkaline phosphatase substrate (KPL) was added to each well and incubated at room temperature in the dark.

Western Blot

The Western-Light™ Chemiluminescent Detection System (TROPIX, Inc.) reagents and methods were used in conjunction with a PVDF membrane, *L. destructor, G. domesticus and A. ovatus* mite homogenates (as described above) and purified mAb.

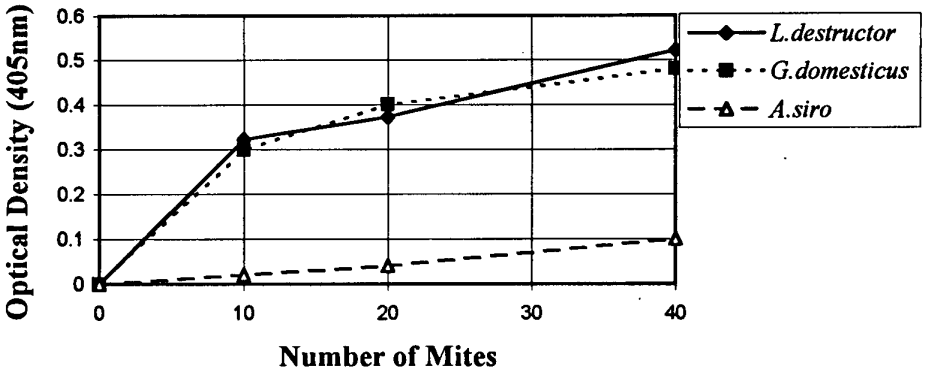
RESULTS

Sensitivity of the mAb 42B6

The sensitivity of the mAb 42B6 to adult *L. destructor, G. domesticus and A. siro* was determined by indirect ELISA. The optimum protein content for the antibody was 1µg/ml. Figure 1 shows that the mAb was selective for the two Glycyphagid species

of mite - *L. destructor* and *G. domesticus*. Calibration curves determined that the assay was sensitive to detect a minimum of one Glycyphagid mite.

Figure 1. Indirect ELISA determining the sensitivity and selectivity of mAb 42B6 (n=5)



Reactivity with other species of mites

The mAb was tested for its reactivity with adults of the following species of storage mites: *L. destructor, G. domesticus, G. ornatus, T. putrescentiae, T. longior, T. palmarum, A. siro, A. farris, D. pteronyssinus, B. tropicalis, C. berlesei* and *A. ovatus.*

The results are presented in Table 1. For the purposes of this study, an optical density value of 0.1 or below was considered negative, as a response in that range is too close to the background readings to be of meaningful significance. Reactivity was strongest with *L. destructor, G. domesticus* and *A. ovatus.* Response was marginal with *T. longior, T. putrescentiae, A. siro, A. farris* and *B. tropicalis.* Reactivity was regarded as negative with *T. palmarum, G. ornatus, C. berlesei* and *D. pteronyssinus.*

Table 1: Reactivity of mAb with mite species in an indirect ELISA (n=5)

Species	Optical Density Value at 405nm
Lepidoglyphus destructor	1.082
Glycyphagus domesticus	0.986
Aleuroglyphus ovatus	0.887
Tyrophagus longior	0.373
Tyrophagus putrescentiae	0.312
Acarus farris	0.265
Acarus siro	0.246
Blomia tropicalis	0.216
Cheyletus sp.	0.103
Caloglyphus berlesei	0.083
Tyrophagus palmarum	0.045
Glycyphagus ornatus	0.030
Dermatophagoides pteronyssinus	0.007

Reactivity with four species of storage insect pest

Reactivity of the mAb was also tested with adults of the following storage insect species: *A. advena, O. surinamensis, S. granarius* and *C. ferrugineus.* There was no significant reactivity with any of these species (Table 2).

Table 2: Reactivity of mAb with storage insects (n=5)

Species	Optical Density Value at 405nm
Oryzaephilus surinamensis	0.025
Sitophilus granarius	0.019
Cryptolestes ferrugineus	0.018
Ahasverus advena	0.010

Reactivity with different life stages of *L. destructor*, dead and live adult mites, and associated materials.

Serological tests were undertaken as before to assess the reactivity of eggs, nymphs, resting tritonymphs, adults which were alive immediately before analysis ("live"). Of the different life stages, adults had the greatest response. Responses to tritonymphs and nymphs were marginal while there was no response with eggs (Table 3).

Table 3: Reactivity with different life stages of *L.destructor* (n=5)

	Optical Density Value at 405nm
Eggs	0.144
Nymphs	0.243
Tritonymphs	0.300
"Live" adults	1.217

Also tested were adults which had been killed within the previous 24 hr ("freshly killed"), adults which had been dead for 10 d ("desiccated"), faeces, spent food of *L. destructor* faeces and culture food (yeast and flour), and food only. Of these, live adult mites, dead adult mites and mite faeces all gave strong responses. Responses to spent food and to faeces and food were marginal, while there was little response to food only (Table 4).

Table 4: Reactivity with live and dead adults, faeces and food components of *L.destructor* (n=5)

	Optical Density Value at 405nm
"Live" adult mites	1.217
"Freshly killed" adult mites	1.098
"Desiccated" dead adult mites	1.111
Faeces only	1.222
Spent food	0.497
Faeces and food	0.428
Food only	0.106

Reactivity with laboratory infested and uninfested grain samples
The ball bearing method of extraction was more efficient than the stomacher in extracting mite antigen, shown by higher protein concentrations in the Biorad tests (141 µg/ml protein with 100 mites in a 5g sample compared to 61 µg/ml using the stomacher) so the ball bearing method was used for all results quoted here.

Initially only tissue culture serum was available so an indirect ELISA was the only possible means of mite detection. This was sufficiently sensitive to detect mites alone but not when either barley (Figure 2) or wheat (Figure 3) was present.

To improve sensitivity, the mAb was purified using a Protein G affinity column. A portion of the purified mAb was conjugated with an alkaline phosphatase enzyme (Pharmacia Linkit™ phosphalink) so that a monoclonal-based DAS ELISA could be performed. However, the DAS ELISA only marginally improved sensitivity of mite infested grain samples (Figures 2 and 3).

Whilst attempting to improve sensitivity, a small quantity of pAb raised against *G. domesticus*, was made available. This allowed for further ELISA techniques to be performed. The TAS ELISA, with its extra amplification step improved sensitivity and quantification curves for adult *L. destructor* were successfully produced in both barley (Figure 2) and wheat (Figure 3).

Western Blot
A band at 39 kDa was found with all three species tested. *L. destructor* and *G. domesticus* had a proportionally larger band than *A. ovatus.*

Figure 2. Comparison of three different ELISA assays with known numbers of mites in barley

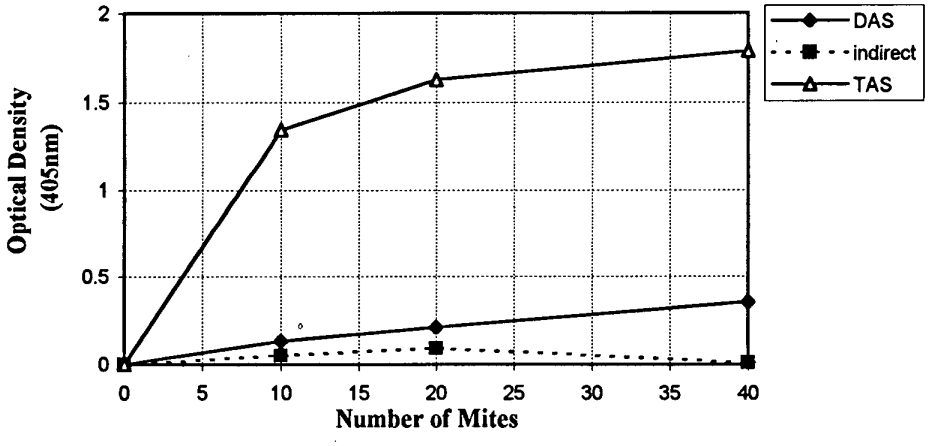
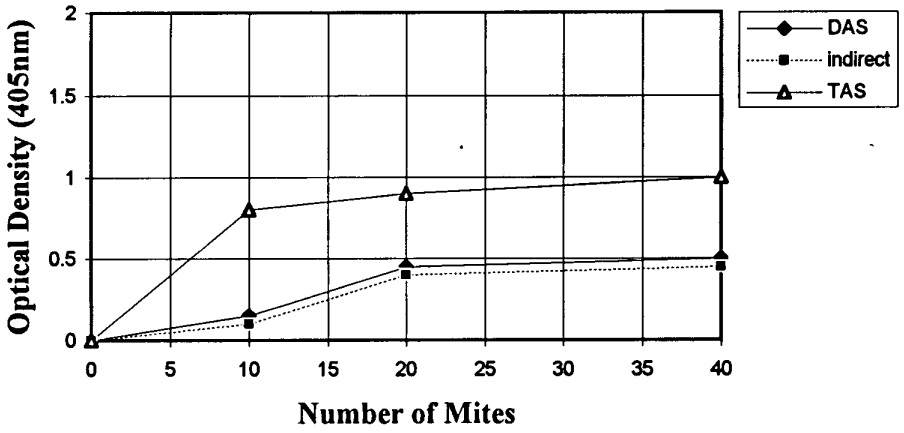


Figure 3. Comparison of three different ELISA assays with known numbers of mites in wheat



DISCUSSION

The mAb used in this study was raised by Härfast *et al.* (1992). Härfast *et al.* (1996) showed that (in the absence of cereal grain) the mAb binds strongly to *G. domesticus* and to a lesser extent to *A. siro* and *T. putrescentiae.* Our results confirm these specificity findings. We also found that there was a strong reaction with *A. ovatus* and a marginal reaction with *T. longior*, *A. farris* and *B. tropicalis.* There was, however, no reaction with the pyroglyphid mite, *D. pteronyssinus* or with the four storage insect species tested. Western Blot analysis was undertaken with the three mite species which showed greatest reaction, *L. destructor, G. domesticus* and *A. ovatus,* to determine whether they share a common protein band. The Western Blot analysis showed there was a band at 39 KDa in all three of these species, which would explain their reactivity with the mAb.

From our work it appears that the amount of antigen present increases through the life stages of *L. destructor*. There was minimal reactivity with eggs but a marginal amount with nymphs and tritonymphs. The antigen is found in largest amounts in both dead and live adults and in their faeces. The presence of the antigen in faecal pellets as well as dead and live *L. destructor* mites suggests that it is likely to be involved in digestion, which supports an earlier deduction by van Hage-Hamsten *et al.* (1992). The antigen can be detected in spent food and faeces plus culture food. Detecting dead mites and mite faeces could be of an advantage for even if live mites are removed from a sample, mite allergens in the product could still be hazardous to health.

The mAb was used to construct calibration curves. This was done with tissue culture serum and mites, in an indirect ELISA. When protein concentrations were over 2 μg/ml, non-specific binding occurred (antigen binding to antigen) decreasing the optical density values. Therefore dilution series for quantification of unknown samples would have to be prepared for accurate results. However, the sensitivity of this method suggests that it would be able to detect mite infestations at very low levels.

The sensitivity of the indirect assay was lower when the mites were crushed with grain. It is known that this mAb detects a 39 kDa allergen in *L. destructor* which is resistant to a variety of treatments. Its resistance to trypsin and protease seems to suggest that the epitope recognised is not proteinaceous but rather of a carbohydrate nature (van Hage-Hamsten *et al.*, 1992). It is therefore possible that the lack of sensitivity in the presence of grain was caused by the mAb being at least partly bound to cereal carbohydrates and therefore unavailable to detect the antigen.

The sensitivity of the DAS ELISA too was lower when the mites were crushed with grain. There are two possible reasons for this. First, the second antibody conjugate used in this assay was the purified anti-39 kDa mAb linked to an alkaline phosphatase enzyme. The laboratory-based Linkit kit may not have been wholly efficient in conjugating enzyme to antibody. There may have been conjugated and non-conjugated antibody in solution but, as only the conjugated antibody will react with the substrate, optical density values would be reduced. Second, the coating mAb could have bound to cereal carbohydrates, blocking binding by the mite antigen.

The TAS ELISA with the use of the anti-15 kDa pAb raised against *G.domesticus* improved sensitivity. The pAb was able to trap out the mite antigen from the plant debris in the sample suspension, and with the extra amplification step, standard curves with relatively good optical densities for both wheat and barley were produced. This demonstrated potential usefulness of an immunoassay for the quantification of *L. destructor* mites in stored grain is a significant advance on previous immunoassays which have focused on detecting house dust and storage mites in dust (Luczynska *et al.*, 1989; Härfast *et al.*, 1996).

The work reported here has proven the potential of the immunoassay method for the rapid and sensitive detection of storage mites. It is clear that the existing antibody is not sufficient by itself for detecting mites in cereal grain and, although we have shown how this can be overcome by using an additional pAb, it would not be without its own limitations. The supply of pAb is not guaranteed and the pAb/mAb assay is limited to the detection of the Glycyphagid mites *L. destructor* and *G. domesticus.*

It is therefore proposed that future work should raise an antibody against a common storage mite protein rather than crude extracts of mites, with the additional precaution that the selection pressure includes lack of reactivity to cereal grains. This would allow detection of a wider range of mite pests even in the presence of grain. Previous experience in related studies suggests that this approach would be highly successful and the best way to exploit the encouraging results obtained in the present study.

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