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PROJECT REPORT No. 42

USE OF ENZYME IMMUNOASSAYS TO QUANTIFY TOTAL FUNGI AND POSSIBLY THE GENERA *ASPERGILLUS, PENICILLIUM* AND *FUSARIUM* IN CEREAL SAMPLES

DECEMBER 1991

PRICE £3.00

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by

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Final report of a one year project at the MAFF Central Science Laboratory, London Road, Slough, Berks., SL3 7HJ. The work commenced in April, 1990 and was supported by a grant of £17,723 from the Home-Grown Cereals Authority (Project No. 0002/1/90).

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ABSTRACT

A double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) was constructed using an antiserum raised against *Penicillium aurantiogriseum* var. *melanoconidium*. The assay protocol was selected and preliminary optimization carried out using a pure preparation of the homologous fungus (i.e. fungus against which antibodies (antisera) had been raised) as test material. Reproducibility was monitored. Intra-plate variation was acceptable but inter-plate variability was poor, suggesting that the method needs further improvement. Preliminary investigations into the sensitivity of the test for other fungi were carried out using mycelial preparations of fungi grown in synthetic liquid medium. Results indicated greater and preferential detection of the three major groups of storage fungi (*Aspergillus, Eurotium* and *Penicillium*) over field fungi. The assay was, however, unable to detect spores grown in the laboratory on synthetic medium. Samples from spiked and unspiked barley were also used as test material. The assay was unable to detect the homologous fungus although present as both mycelium and spores from spiked barley. Further, the results showed that a high cross-reactivity was apparent with unspiked barley extracts.

The work carried out has allowed basic assay parameters and methodology to be set but clearly the assay requires further refinement in order to identify fungi from commodity. Further work is necessary to identify more suitable antibodies for utilization in the DAS ELISA and/or to identify other assay designs which work better than the DAS ELISA.

OBJECTIVE

To develop a prototype DAS ELISA for the detection of storage fungi in cereals.

INTRODUCTION

The agricultural industry requires a rapid method for identifying fungal contamination of raw foodstuffs. Moulds are widespread environmental contaminants that, given the right growth conditions, not only lead to spoilage and economic loss but can also represent a health hazard due to the production of mycotoxins (Elling *et al.*, 1975, Umeda, 1977). Methods for the detection of moulds in food have been reviewed by Jarvis *et al.*(1983). These include the Howard Mould Count, fluorescence microscopy, direct microscopic analysis, direct plating and propagule counts. Alternative techniques that are also cited as applicable to the analysis of food are chitin assay, measurement of microbial ATP and electrical measurement.

Currently the most widely used approach for enumeration and identification of moulds in cereals is dilution plating. Such a traditional culture method is time consuming (5-14 days) and if identification is required beyond genus level highly skilled personnel are required. The inherent delay in this method makes it particularly unsatisfactory when a result is required urgently. Cultural methods also possess mycological limitations of which the best recognised is a tendency to over-estimate fungi which produce large numbers of spores on the substrate. Other disadvantages are that accuracy is anything from 20-50% and that only living fungi are estimated. The alternative chemical methods are laborious or need complicated expensive laboratory equipment.

For overcoming such problems immunological techniques are increasingly being viewed as promising, since results can be provided much more quickly and cheaply and with less expertise. Immunoassays have been widely used in the medical field for diagnosis of fungal diseases such as aspergillosis and have also been applied in the field of fungal disease of plants (Clarke *et al.*, 1986). Increasingly they have been applied to the detection of moulds in foodstuffs (Notermans *et al.*, 1986a, 1986b, Notermans and Kamphuis, 1990, Lin *et al.*, 1986, Mohan,1988, Kamphuis *et al.*, 1989). Less interest has been directed towards cereals although Dewey *et al.* (1990) have developed immunoassays for *Penicillium islandicum*, a contaminant primarily of rice grain.

At MAFF, Central Science Laboratory, government funding has supported the raising of antisera (polyclonal antibodies) against *Penicillium aurantiogriseum* var. *melanoconidium, Eurotium amstelodami* and *Fusarium culmorum*. Monoclonal antibodies have also been raised to the *Penicillium* species. One of these antisera has been shown to be extremely cross-reactive with 27 storage fungi and ten field fungi (Banks *et al.*,1991) and was consequently suitable for use in a broad spectrum DAS ELISA for the detection of fungi in stored cereals. Accordingly, it was decided that the following stages would comprise a study to develop such an assay:-

a) Optimization of DAS ELISA method.
b) Sensitivity of DAS ELISA for heterologous fungi.
c) Ability of DAS ELISA to detect spores.
d) Testing of barley samples: unspiked and spiked.

MATERIALS AND METHODS

ANTISERUM PRODUCTION

Antisera to *Penicillium aurantiogriseum* var. *melanoconidium* had been previously raised in Simone Noir rabbits with MAFF funding. The full method and cross-reactivity of the unprocessed serum for other field and storage fungi is reported in Banks *et al.*(1991).

SAMPLE PREPARATION

Pure laboratory antigen preparations of soluble mycelial extracts of field and storage fungi: All the fungi tested (Table 2) were grown up from freeze dried phials held in the department's culture collection. The *Fusarium* species were grown on potato sucrose agar (Booth, 1971); *Eurotium* species and *Wallemia sebi* on 2% malt agar with 10% salt; and all others on 2% malt. Spore suspensions were prepared in 0.01% Tween 80 (British Drug Houses) and after three washings by centrifugation 1ml of spore suspension at 10^6 ml^{-1} was inoculated into 100ml liquid medium (Burrell *et al.*, 1966). The medium for *Eurotium* and *Wallemia* was supplemented with 100g salt per litre of medium. Flasks were shaken on a rotary shaker (L H Engineering Ltd., Slough) at 140 rpm, for 7 d in the dark at 25°C.

The mycelial material formed was filtered into a sintered glass funnel (Grade 3, Gallenkamp) and washed with sterile distilled water, then sterile phosphate buffered saline (PBS), the filtrate being discarded. It was frozen overnight at -20°C and then freeze dried (Modulyuo 4K, Edwards). Following snap freezing with 50ml of liquid nitrogen the material was homogenized at full speed for 1 min in an Omni-mixer blender (Camlab). A pestle and

mortar was then used to further grind the material to a fine powder. This was then resuspended in PBS (200mg of fungus in 1.5ml PBS).The suspension was centrifuged at 3000g for 10 min at 4°C. Following centrifugation the supernatant was drawn off and used as antigen (sample) in the DAS ELISA. Protein assays were carried out using a modified Bradford protein assay (Banks *et al.*,1991) on each of the antigens in order that the DAS ELISA's sensitivity for each fungal antigen could be assessed.

Spore suspension preparation: *Penicillium aurantiogriseum* var. *melanoconidium* was subcultured onto a 2% malt agar slope. Following incubation at 25°C for 7 d, 5ml of sterile PBS with 0.01% Tween 20 (PBST) was added to the slope and the spores scraped off the agar with a wire loop. To ensure even mixing the bottle containing the slope was placed briefly twice, on a vortex mixer. Ten-fold dilutions of the spore suspension were then prepared in PBST and 0.1ml of each dilution (10^{-3} to 10^{-8}) was spread over 2% malt agar plates in triplicate. The plates were incubated at 25°C for 8 days before colony counts were made. The spore dilutions were also used as test material on the DAS ELISA performed the same day.

Unspiked barley samples: Different methods of barley sample preparation were tried as detailed in Table 4 (Appendix). Following homogenization 10-fold dilutions of the supernatant were prepared in PBST and a DAS ELISA was carried out.

Spiked barley samples: Flasks containing 100g of autoclave sterilized barley with a moisture content adjusted to 30% were inoculated with *P.aurantiogriseum* var. *melanoconidium*. The inoculum was provided from a 2% malt slope. A dry loopful of spores was transferred to each flask of barley and distributed evenly about the surface by touching at various points. Flasks were incubated at 25°C. A flask was removed daily and stored at -80°C thus giving samples with variable mould growth. A control uninoculated flask of barley was also included.

10g of material from the control, day 3 and day 7 flasks was removed and soaked overnight in 50ml sterile PBST at 4°C. It was next homogenized for 5min in a stomacher. The large debris was then allowed to settle (2 min). The resulting supernatant was used to prepare 10-fold dilutions in filtered PBST with 3% bovine serum albumin (BSA) over the range 10^{-3} to 10^{-8}. Using these dilutions spread plates in triplicate were set up for each sample applying 0.1 ml/plate. The medium used was Dichloran Rose Benegal Chloramphenicol (DRBC, Oxoid). A DAS ELISA was also carried out on the diluted material.

ELISA PROCEDURE

A DAS ELISA procedure (Voller *et al.*, 1979) was used.

Purification and conjugation to horse radish peroxidase (HRPO) of immunoglobulin (Ig): 9ml of antiserum was precipitated with 2.25g ammonium sulphate over 24 hours. The precipitate was then washed with 1.75M ammonium sulphate and taken up into a smaller amount of water before being dialysed into sodium acetate buffer pH 5.0. The material was then centrifuged at 2000g for 10 min and the resulting supernatant reduced to approximately 10ml by dialysis against polyethylene glycol. Further purification was carried out on DEAE Sephadex A50 (anion exchange column). Fractions eluted with sodium acetate buffer pH 5.0 were collected and bulked. Purified material for use as the 'trapper' first stage in the DAS ELISA was dialysed into PBS pH 7.2. Protein concentration was determined at A_{280} and this material was stored in aliquots at -20°C. Material for conjugation to HRPO was dialysed against carbonate buffer pH 9.6. The concentration of Ig was then adjusted to 8mg/ml by

diluting in carbonate buffer following protein concentration determination at A_{280}. The Ig was then conjugated to HRPO by the periodate method (Nakane and Kawaoi, 1974, Tijssen and Kurstak, 1984) at a rate of 4mg HRPO to 8mg Ig. Following conjugation the conjugate was diluted 1:1 with glycerol and stored at -20°C.

Microtitre plate coating with 'trapper' Ig: Purified Ig was diluted in carbonate buffer pH 9.6 to give 20µg/ml. Fifty microlitres were added to each well of the microtitre plate (Immulon 1,Dynatech), which were then incubated for 2 h at 25°C in a moist box. Following incubation the plates were washed three times with PBST, 200µl/well. Each well was next blocked with 250µl PBST containing 3% BSA overnight at 25°C in a moist box. After blocking the plates were washed twice as before, blotted dry and stored at -20°C in a sealed container.

Assay: Ig-coated microtitre plates were removed from -20°C and allowed to warm to room temp before being washed once with PBST. Dilutions of the samples (as detailed in methods or results section) were prepared in PBST with 3% BSA. Quantities of 50µl were added in triplicate to wells for each sample and the plates incubated in a moist box for 2 h at 25°C. After incubation the plates were washed four times with PBST. Conjugate prepared as above and diluted 1:1000 in PBST with 3% BSA was then added to each well (50µl/well).The plates were incubated for 1 h at 25°C in a moist box before washing as previously. Finally,100µl tetramethylbenzidine (120mg in 20ml dimethyl sulphoxide, diluted 1:62.5 in sodium acetate buffer pH 5.5 with 0.004% hydrogen peroxide) was added to each well. Following incubation in the dark for 15 min at 25°C the reaction was stopped with 50µl/well 4N sulphuric acid. The absorbance was measured at 450nm.

RESULTS

OPTIMIZATION OF DAS ELISA PROCEDURE

Early in the construction of the DAS ELISA inadequate blocking of unbound sites on the microtitre plates gave rise to high nonspecific background absorbance readings due to conjugate binding directly to the plate. This was reduced by introducing PBST with 3% BSA as a diluent throughout, reducing conjugate incubation time from 2 h to 1 h and selection by chequer board titration of a conjugate dilution that minimized nonspecific interference but that remained strong enough to distinguish adequately positive and negative samples. A chequer board titration was also carried out to determine the optimal 'trapper' concentration which was confirmed at 20µg/ml.

Throughout the following experiments reproducibility of the finalized method was monitored using a pure soluble mycelial extract of *P. aurantiogriseum* var. *melanoconidium*, the results of which are presented in Table 1 (Appendix). Coefficients of variation (CVs) have been calculated for both intra- and inter-test variability. The intra-test variability was based on the use of triplicates for each sample and on the whole reproducibility was quite good giving most CVs below 10. However, at the lower concentration (1µg/ml) the reproducibility was noticeably poorer on two occasions. The inter-test variability was greater, CVs ranging from 15.00 to 25.00, again the lower concentration being the least reproducible.

SENSITIVITY OF DAS ELISA FOR HETEROLOGOUS FUNGI

The sensitivity of the DAS ELISA technique for 9 field fungi and 27 field fungi was assessed. Figures for the lowest level of detection are detailed in Table 2 (Appendix). From the results

it can be seen that the test was able to detect storage fungi, particularly most *Aspergillus, Eurotium* and *Penicillium* species to low levels (except *P. rubrum* and *E. rubrum*), and was less able to detect field fungi. In all cases except for *Cladosporium cladosporioides* and *Hyalodendron sp.* greater than 1000µg/ml was required to detect the field fungi.

ABILITY OF DAS ELISA TO DETECT SPORES

The antiserum used for the DAS ELISA was raised against a soluble mycelial extract of *Penicillium aurantiogriseum* var. *melanoconidium*. The ability of the assay to detect spores of the same species was investigated. Dilution plating was carried out for comparison with the DAS ELISA. The results of both are presented in Table 3 (Appendix). Although colonies grew from a range of dilutions the only DAS ELISA reading was gained at a dilution of 10^{-1} and this was low (0.26).

BARLEY SAMPLE PREPARATION

The most appropriate method for barley sample preparation was identified following an exercise in which different methods of homogenization were compared, with the resulting supernatants being used as test material in the DAS ELISA. The results are given in Table 4 (Appendix). A high degree of nonspecific interaction for all samples of barley was shown when used neat, even those unhomogenized, as evinced by absorbance values of greater than 0.20. The homogenized samples gave highest nonspecific binding which suggests that internal components as well as surface components of the barley cross-react with the antibody used in the test. However, with increasing dilution,the nonspecific interaction is reduced, so dilutions starting at 10^{-3} were used in the spiked barley experiment.

DETECTION OF *PENICILLIUM AURANTIOGRISEUM* VAR.*MELANOCONIDIUM* FROM BARLEY

A comparative exercise in which the DAS ELISA was run alongside dilution plating was carried out using uninoculated barley and two inoculated barley samples. The counts for the samples are presented in Table 5 (Appendix) along with the corresponding DAS ELISA absorbance reading for each dilution of sample used. Results indicate that despite high levels of contamination as demonstrated by the dilution plate results the DAS ELISA was unable to detect fungus from grain.

DISCUSSION

Previous work carried out at CSL Slough funded by the MAFF Chief Scientist's Group has yielded a rabbit antiserum which was shown to be extremely cross-reactive for a variety of field and storage fungi (Banks *et al.*, 1991). The next step, therefore, was to assess this antiserum's suitability as a reagent in a broad spectrum immunoassay to detect field and storage fungi from stored commodities such as barley. The DAS ELISA was selected as a suitable assay design based on its relative ease of construction and performance.

From the results several conclusions can be made. Firstly, despite the unpurified antiserum cross-reacting with a large number of both field and storage fungi, the purified immunoglobulin was less reactive (Table 2). When used as first stage 'trapper' immunoglobulin and conjugate in the DAS ELISA the cross-reactivity of the immunoglobulin with field fungi was virtually non-existent. This indicates that during the purification and/or conjugation steps the reactivity of the immunoglobulin is reduced. This obviously needs to

be borne in mind in the future when selecting and purifying other antisera or monoclonal antibodies, and reinforces the need to check cross-reactivity of purified immunoglobulin despite this perhaps having already been carried out on the original antiserum.

Despite the ability of the assay to detect pure preparations of different fungi, the experiment with contaminated barley demonstrated that the assay was unable to detect fungi grown on commodity. The experiment in which spores were used as test material had given a preliminary indication that the assay was unable to detect fungal spores but the barley extracts contained a mixture of mycelium and spores, so some response was expected. Why the assay was unable to detect anything is difficult to determine without further experimental work. However, possibly the mycelial components were too dilute or were not adequately released by the homogenization procedure employed. Although the work with pure laboratory grown cultures of *Penicillium aurantiogriseum* var. *melanoconidium* showed a detection as low as 0.1µg/ml (5ng/well) protein, the relationship of this figure to colony counts is unknown. It could therefore be that although there was material present to promote colony growth the protein concentration was below the threshold of detection. An alternative reason for lack of detection may be that the antigens produced in synthetic liquid medium used to raise the antiserum may be absent when the fungus is grown on a different medium such as barley. Either way in its present form the assay is unable to detect fungi from stored commodity even at very high levels of obvious contamination.

Although the assay was unable to complete the function required of it, three other useful pieces of information were generated. High cross-reactivity was encountered with uncontaminated barley extracts which is undesirable. This means that dilutions of homogenates have to be used thus perhaps running the risk of diluting out the fungal antigens if they are occurring at low concentration. It is clear that in the future antisera or monoclonal antibodies need to be screened against extracts of commodities likely to be encountered. Only those that show no or very limited cross-reactivity should be selected for use in immunoassay development.

Secondly, the DAS ELISA's reproducibility was monitored using a pure preparation of *Penicillium aurantiogriseum* var. *melanoconidium*. The acceptable CVs gained for the intra-test variability suggest that the wells are being evenly coated and that application of reagents and washing procedures are consistent. However, the higher CVs gained for inter-test variability suggest that improvements to the technique are necessary. Major causes of variance may be the deterioration of the immunoglobulin-coated plates which are stored at -20°C or batch to batch variation of buffers and diluted reagents. To improve reproducibility, coating of plates and fresh preparation of all buffers the day before required may be necessary to prevent deterioration of both as a result of storage. Additionally more vigorous washing and monitoring of temperature variation within the laboratory may improve inter-test reproducibility.

Thirdly, this work has identified that a difference exists between mycelium and spores, such that an antiserum raised against a mycelial extract of fungus is unable to detect spores of the same fungus. This information should be considered when raising further antisera or monoclonal antibodies. Since the likely test material will be a mixture of spores and mycelium, antisera or monoclonal antibodies should perhaps be raised against a cocktail of both fungal components.

It has to be concluded that at the present time an assay to replace dilution plating remains unavailable. The present project, however, has permitted basic DAS ELISA assay parameters

and other methodologies to be set. It highlighted several potential problematic areas (i.e. cross-reactivity of antifungal antibodies with uninfected commodity, inter-test variability and non-detection of spores by an antiserum raised against mycelium) which should assist assay development in the future.

ACKNOWLEDGEMENTS

The author acknowledges the valuable contributions made to this piece of work by J H Clarke, J N Banks, R H Shamsi, J M Naylor and B J Northway without whom the antiserum would not have been characterized, purified or conjugated nor fungal antigens been available as test material.

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GLOSSARY

ANTIBODY: Antigen specific protein synthesized in an animal in response to entry of a foreign substance (eg. fungi).

ANTIGEN: Any substance which elicits a specific immune response (eg. fungi).

ANTISERUM: Serum containing antibodies against a known antigen. Acts as a source of polyclonal antibodies.

CHEQUER BOARD TITRATION: Method of establishing correct working dilution of reagents by titrating reagents against each other in a microtitre plate. One reagent is diluted horizontally and the other vertically so wells occur with all dilution combinations.

DOUBLE ANTIBODY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (DAS ELISA): Method for detecting antigen (eg. fungi). Fungus-specific immunoglobulin is immobilized onto a solid surface (eg. microtitre plate). Antigen then applied followed by further fungus-specific immunoglobulin tagged with enzyme.

ENZYME IMMUNOASSAY: Assay where either antibody or antigen are tagged with an enzyme to form a conjugate, which has both enzymatic and immunologic reactivity.

IMMUNOGLOBULIN (Ig): Protein that has antibody activity.

MONOCLONAL ANTIBODY: Antibody derived from a single clone of antibody forming cells.

POLYCLONAL ANTIBODY: Mixture of antibodies derived from many different clones of antibody forming cells.

APPENDIX

TEST			[PROTEIN] µg/ml			
REF	100		10		1	
	A^{450}	CV	A^{450}	CV	A^{450}	CV
SJC491	0.53	2.33	0.34	0.74	0.19	7.96
SJC591	0.52	8.23	0.38	2.86	0.25	1.83
SJC691	0.73	3.88	0.56	3.54	0.34	20.49
SJC791	0.68	4.17	0.47	2.67	0.20	8.89
SJC991	0.53	5.10	0.38	11.60	0.20	20.42
CV	15.00		19.00		25.00	

TABLE 1: DAS ELISA ABSORBANCE VALUES FOR *PENICILLIUM AURANTIOGRISEUM* VAR. *MELANOCONIDIUM* STANDARDS PERFORMED ON DIFFERENT DAYS

FUNGI	LOWEST LEVEL OF DETECTION OF PURE ANTIGEN (BASED ON [PROTEIN] µg/ml
FIELD FUNGI	
Alternaria alternata	> 1000
Aureobasidium pullulans	> 1000
Cladosporium cladosporioides	100
Cladosporium herbarum	> 1000
Fusarium avenaceum	> 1000
Fusarium culmorum	> 1000
Fusarium moniliforme	> 1000
Hyalodendron sp.	1000
Verticillium lecanii	> 1000
STORAGE FUNGI	
Apiospora montagnei	> 1000
Aspergillus candidus	10
Aspergillus flavus	1
Aspergillus nidulans	100
Aspergillus ochraceus	0.1
Aspergillus restrictus	0.1
Aspergillus terreus	10
Aspergillus versicolor	0.01
Byssochlamys nivea	100
Eurotium amstelodami	1
Eurotium chevalieri	0.1
Eurotium repens	100
Eurotium rubrum	> 1000
Monascus ruber	100
Mucor racemosus	> 1000
Paecilomyces variotii	> 1000
Penicillium aurantiogriseum	1
Penicillium aurantiogriseum var. melanoconidium	0.1
Penicillium cyclopium	0.1
Penicillium hordei	0.1
Penicillium martensii	1
Penicillium notatum	10
Penicillium roquefortii	10
Peniciliium rubrum	> 1000
Scopulariopsis brevicaulis	> 1000
Trichothecium roseum	100
Wallemia sebi	> 1000

TABLE 2: LOWEST LEVEL OF DETECTION BY DAS ELISA OF A VARIETY OF FUNGI LIKELY TO BE ENCOUNTERED IN STORED GRAIN

DILUTION OF SPORE SUSPENSION	COLONIES/0.1ML	DAS ELISA A^{450}
10^{-1}	>100	0.26
10^{-2}	>100	0
10^{-3}	>100	0
10^{-4}	>100	0
10^{-5}	88	0
10^{-6}	5	0
10^{-7}	0	0
10^{-8}	1	0

TABLE 3: COMPARISON OF DAS ELISA WITH DILUTION PLATING USING A SPORE SUSPENSION OF *PENICILLIUM AURANTIOGRISEUM* VAR *MELANOCONIDIUM*

SAMPLE	METHOD OF PREPARATION	DILUTION	A_{450}
1	10g barley + 50ml PBS	neat	0.94
	+ 3% BSA	1:10	1.15
	Soaked overnight 4°C	1:100	0.21
	Stomacher - 5 min	1:1000	-0.04
2	10g barley + 50ml PBS	neat	1.23
	+ 3% BSA	1:10	0.72
	Soaked overnight 4°C	1:100	0.10
	Omnimixer (power 7) - 2 x 30s	1:1000	-0.02
3	10g barley + 50ml PBS	neat	0.95
	+ 3% BSA	1:10	0.18
	Soaked overnight 4°C	1:100	0.01
	Handshaken - 60s	1:1000	-0.04
4	10g barley + 50ml PBS	neat	1.18
	+ 3% BSA	1:10	0.73
	Unsoaked	1:100	0.06
	Omnimixer (power 7) - 4 x 30s	1:1000	-0.03
5	10g barley + 50ml PBS	neat	0.39
	+ 3% BSA	1:10	0.07
	Unsoaked	1:100	-0.02
	Handshaken	1:1000	-0.01

TABLE 4: DIFFERENT METHODS OF BARLEY PREPARATION WITH CORRESPONDING DAS ELISA ABSORBANCE VALUES

SAMPLE	DILUTION	COLONIES/0.1ML	DAS ELISA A_{450}
CONTROL	10^{-3}	0	0.07
	10^{-4}	0	0.03
	10^{-5}	0	0.05
	10^{-6}	0	0.06
	10^{-7}	0	0.05
	10^{-8}	0	0
DAY 3	10^{-3}	18	0.12
	10^{-4}	2	0.05
	10^{-5}	0	0.05
	10^{-6}	0	0.06
	10^{-7}	0	0.06
	10^{-8}	0	0.03
DAY 7	10^{-3}	>100	0.03
	10^{-4}	>100	0
	10^{-5}	70	0
	10^{-6}	6	0
	10^{-7}	0	0
	10^{-8}	0	0.02

TABLE 5: COMPARISON OF A DAS ELISA WITH DILUTION PLATES USING UNSPIKED AND SPIKED BARLEY AS TEST MATERIAL