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Determination of Alt a 1 (*Alternaria alternata*) in poultry farms and a sawmill using ELISA

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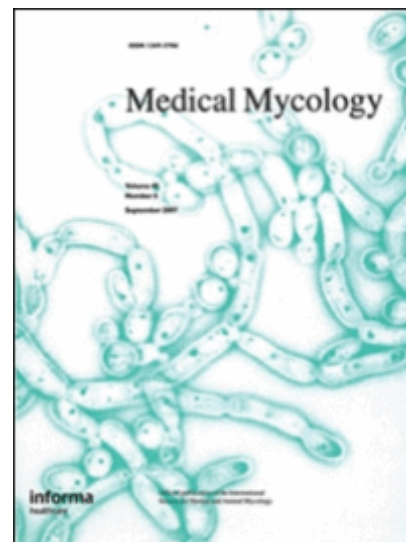
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Abstract

Farm and sawmill workers are exposed to high levels of allergenic fungi, such as *Alternaria alternata*, which are associated with respiratory diseases and asthma.

The aim of this study was to measure the concentration of Alt a 1, a major allergen of *A. alternata*, in indoor dust samples collected in poultry farms and a sawmill using a monoclonal antibody-based enzyme immunoassay. A total of 45 dust samples were collected in poultry farms (30) and the sawmill (15) in Zagreb County (Croatia). The Alt a 1 allergen was detected in all dust samples (100%) collected in three poultry farms. The levels of Alt a 1 were in the range of 0.1-14 µg/g, and the median value was 0.37 µg/g. About 86% of dust samples contained Alt a 1 in the range of 0.1-1.0 µg/g. In the sawmill, no detectable level of Alt a 1 was found (limit of detection=0.12 µg/g). This study has shown that occupational exposure to Alt a 1 allergen in poultry farms deserves monitoring.

Keywords: *Alternaria alternata*; Alt a 1; poultry farms; sawmill; monoclonal-based ELISA

Introduction

Settled dust is often used to assess exposure to indoor allergens (house dust mites, cockroach, dog, cat) ¹. Recently, such studies were employed to evaluate contact with *Alternaria alternata* allergens in house dust samples ^{2 3}. *A. alternata* is a potent allergenic fungus and a number of its allergens have been isolated from the spores and hyphal fragments ^{2 4 5}. Its major allergen is a dimer, Alt a 1, with a molecular weight of 29-31 kD ⁶. There is increasing evidence that *A. alternata* is the most important fungus causing allergic diseases, and sensitization to Alt a 1 is associated with the exacerbation of

asthma 2 5-9. Alt a 1 (purified from allergen extracts by affinity chromatography and ion exchange chromatography) has caused reactions in > 90% of *Alternaria* sensitive individuals 10. *Alternaria* spores are aeroallergens widely distributed in many temperate regions including Croatia 11-13, and their numbers increase significantly in the summer 12-15. Although indoor fungal allergen levels tend to reflect those found outdoors, fungal spores can colonize the indoor environment and thereby significantly affect exposure levels 16. However, few studies have examined the levels of Alt a 1 and other fungal allergens in various indoor environments.

Previous studies have demonstrated that structures that house different animals (poultry, swine, dairy) represent environments with significant levels of bioaerosols containing bacteria, endotoxins, and allergenic fungi (*Alternaria* and *Aspergillus* spp.) 17-21. Several studies reported that farm workers might be at high risk for the development of occupational airway diseases 20 22. Similarly, sawmill workers are also chronically exposed to high levels of allergenic fungi, which can result in respiratory diseases and asthma 23 24. Exposure to fungi has traditionally been assessed by microscopic spore count and culture-based techniques 25. However, there are several immunoassays to quantify environmental *A. alternata* antigens using polyclonal (pAb) or monoclonal antibodies (mAb) 26. Capture enzyme-linked immunosorbent assay (ELISA) for quantitative determination of Alt a 1 using monoclonal antibodies is highly specific for this antigen 26-28, although cross-reactivity with related fungi could not be completely excluded 29. However, the limitation of this assay is that provides a low frequency of detection of Alt a 1 in house dust samples (<25%), which in turn, limits its application in the monitoring of residential environments 26 30.

To our knowledge, capture mAbs ELISA has not yet been validated for determining dust-borne Alt a 1 level in occupational settings with high bioaerosol levels, including allergenic fungi. Using this monospecific assay for quantifying Alt a 1 in animal farming environments could help to provide a greater understanding of the relationship between dose-related fungal allergen exposure and adverse health effects 31. Therefore, the aim of this pilot study was to determine Alt a I levels in settled dust samples collected from poultry farms and from a sawmill in Zagreb County (Croatia). Samples were recovered during daily routine activities of workers, and assayed using mAbs based ELISA. The analytical validation of the assay included intra-and inter-assay precision, accuracy, and sensitivity.

Materials and methods

Dust collection and extraction

Thirty settled dust samples were collected from poultry farms, and 15 from a sawmill, all located in Zagreb County (Croatia). The samples from the poultry farms were collected in the early spring (Feb/March) of 2007 from the floors and several horizontal surfaces. Laying hens in these poultry farms were bred either in cages (farms 1 and 2) or in cage-free conditions (farm 3). All dust samples were collected in the morning (8:00-9:00 a.m.), and were placed in sterile plastic bags and stored at -20°C until used for extraction.

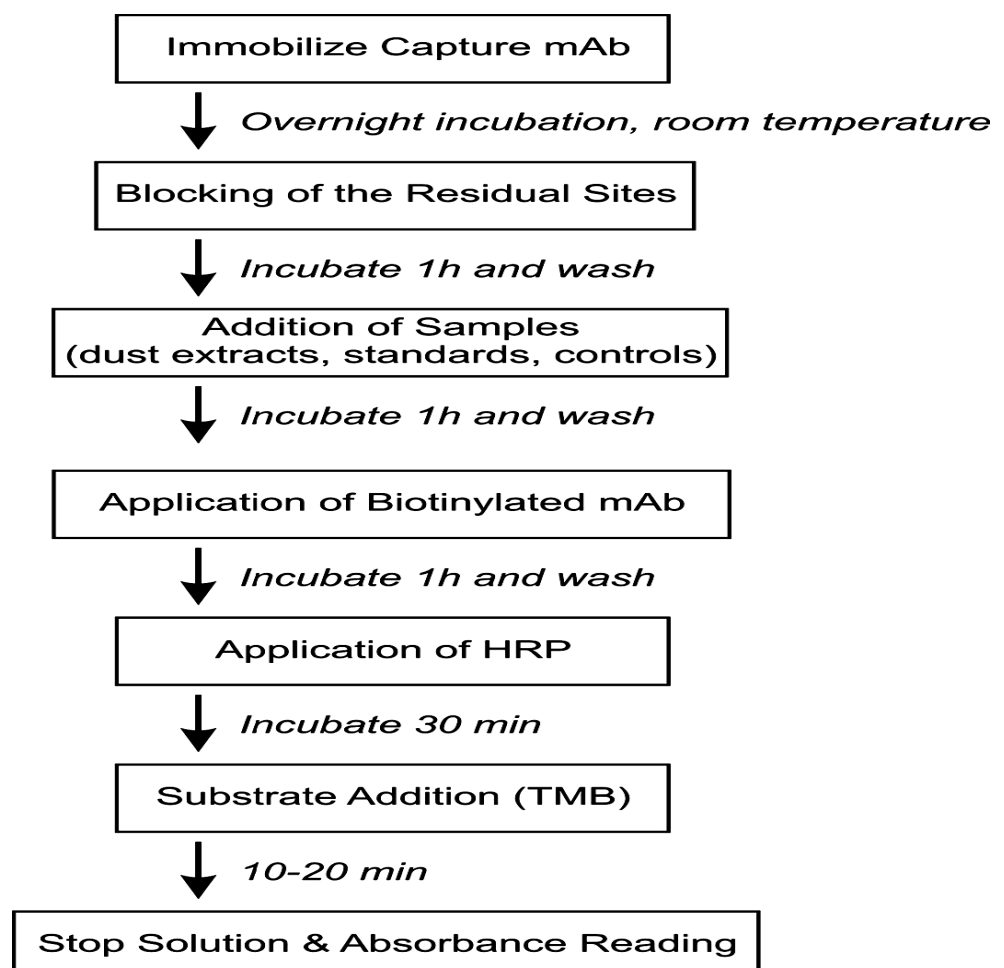
In the sawmill which is used to process deciduous timber (oak, beech, and ash), the dust samples were collected from different production stages in July, 2007. Samples were placed into sterile plastic bags and stored at -20°C until extraction and analysis.

Before the extraction, large debris was removed from the samples using a 250 µm sieve. A 100-mg portion of a sample of fine dust was mixed with 1 ml of PBS-T (phosphate buffer saline with 0.05% Tween 20, pH 7.4) using a wrist-action shaker (Ika, Vortex, Germany) at room temperature for 1 h and then centrifuged ³². The supernatants were stored at -20°C until analysis for Alt 1 content.

ELISA

The concentrations of Alt a 1 were determined by using a commercial ELISA kit (Indoor Biotechnologies Ltd, Cardiff, UK) with monoclonal antibodies, and recombinant Alt a 1 standard (r Alt a 1) as described previously ^{27 28 33 34}. Mouse anti-Alt a 1 mAb (clone 121 G5 G8, isotype IgG2A, lot 29034) was employed as the catching antibody, and biotinylated mouse anti-Alt a 1 mAb (clone 121 G5 G8, isotype IgG1, lot 2851) as the secondary antibody. The assay is unique in that a single mAb is used as both capture and biotinylated secondary antibody. Purified recombinant Alt a 1 (r Alt a 1) was used (1,000 ng Alt a 1/ml, lot 2729) as a reference standard. Maxisorp 96-well ELISA plates (Nalge-Nunc International, Rochester, NY) were coated with 100 µl of mAb 121 (10 µl/10 ml of carbonate-bicarbonate buffer, pH 9.6) by overnight incubation at room temperature (Fig. 1). Excess antigen was removed with PBS-T, and the plates were blocked with 300 µl of 1% BSA-PBS-T (bovine serum albumin, Sigma, USA) for 1

h. The wells were aspirated and washed with PBS-T. One hundred microliters of standard, samples, blank, and control samples were added to respective wells and incubated for 1 h. Sample extracts were diluted three times, or more if required, using 1% BSA-PBS-T. Serial dilutions of the standard (r Alt a 1) (from 16-0.06 ng/ml) were used to make a standard curve. After washing with PBS-T, the biotinylated mAb (10 µl per 10 ml of BSA-PBS-T) was added to the wells. After an additional 1-h incubation period, the wells were aspirated again, washed with PBS-T, and bound biotinylated mAb was detected using streptavidin-horseradish peroxidase (HRP) as the detecting reagent and TMB (3,3',5,5'-Tetramethylbenzidine) as the substratum (Adaltis, Italy). HRP (100 µl) was added to all wells and incubated for 30 min. After washing with PBS-T, TMB substrate solution (100 µl) was added to all wells. The plates were incubated for 20 min, and the reaction was quenched by the addition of 100 µl of sulphuric acid (1 M) as a stop solution. The optical absorbance of each well was read at 450 nm using a microplate reader (PersonalLab, IASON, Graz, Austria). ELISA assays was performed at room temperature. All results are the average of three replicate analyses and are expressed as µg/ g of sieved dust.



[\[Enlarge Image\]](#)

Fig. 1. Flow diagram showing capture ELISA assay for determination of Alt a 1 in dust samples.

Assay characteristics

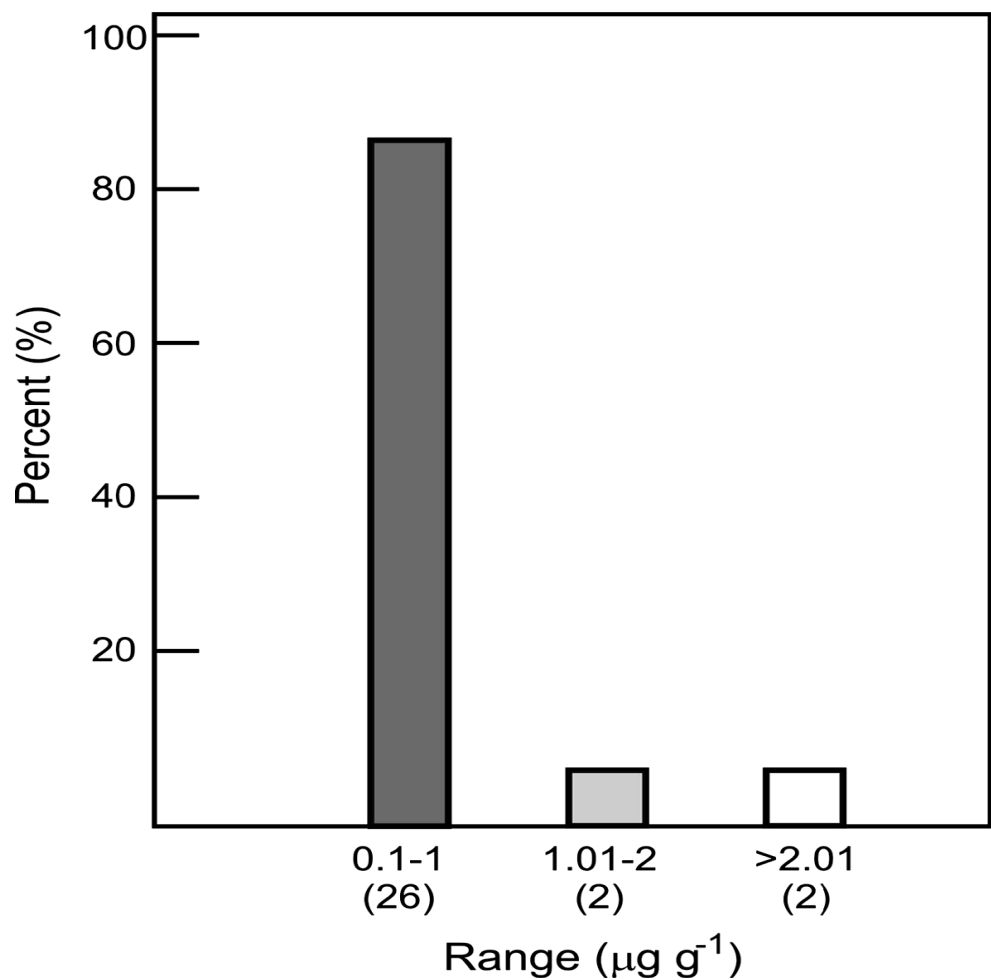
Intra-assay precision was calculated from daily measurements ($n=20$) of one control extract containing low Alt a 1 levels. Inter-assay precision was determined by the repeated determination of the same control extract over five days (four repetitions a day). The results are expressed as coefficient of variation CV (%). The accuracy of measurements was evaluated from five repeated analyses of one dust extract (with low allergen levels) spiked with the reference standard. The results were expressed as the percent of the expected value. The limit of detection of the assay was calculated as the ratio of 3.3 standard deviation of the blank ($n=20$) and the slope of the calibration curve.

Statistical analysis

Statistical calculation was performed using the Statistica for Windows Release 5.5, StatSoft Inc. All data of Alt a 1 in dust extracts were tested for normal distribution with the Kolmogorov-Smirnov test. The data are asymmetrically distributed, and the results are presented as median, 25th-75th percentile (interquartile range), and range (minimum-maximum). Mann-Whitney tests were used to compare Alt a 1 levels between poultry farms. The level of significance was set at $P<0.05$.

Results

Alt a 1 was detected in all dust samples ($n=30$) collected at the three poultry farms (100%). Its mass fractions (median, interquartile ranges and ranges) for each poultry farm (farms 1-3) are shown in [Table 1](#). No statistical difference was found among the median Alt a 1 in dust samples from farms 1, 2, and 3 ($P>0.05$). The overall median dust level of Alt a 1 was 0.37 $\mu\text{g/g}$ (range 0.12-14 $\mu\text{g/g}$). About 86% of dust samples were found to contain Alt a 1 levels in the range of 0.1-1.0 $\mu\text{g/g}$ ([Fig. 2](#)). Alt a 1 ranged 1-2 $\mu\text{g/g}$ in only two samples (7%), and was $> 2 \mu\text{g/g}$ in another two samples (7%).



[Enlarge Image]

Fig. 2. The percentage (%) of dust samples ($N=30$) from poultry farms where the Alt a 1 concentrations ranged in three levels: 0.1-1 µg/g, 1.01-2 µg/g, and > 2.01 µg/g. The number of participants in each group is indicated in parentheses along the abscissa.

Table 1 Median, percentile and range of Alt a 1 (µg/g) in dust samples (n=30) from three poultry farms near Zagreb

	Median	25 th	75 th	Range	<i>n</i>
Farm 1	0.45*	0.24	0.84	0.14-14	18
Farm 2	0.33*	0.18	0.48	0.12-0.71	7
Farm 3	0.29*	0.24	0.59	0.15-0.61	5

*($P>0.05$).

Alt a 1 was not detected in any of the 15 dust samples collected in the sawmill (< 0.12 µg/g, limit of detection).

Intra- and inter-assay precision for Alt a 1 were 5.3 and 12.2%, respectively, and the accuracy of Alt a 1 was 93%.

Discussion

In our study, Alt a1 levels were measurable and similar in all poultry farms, which suggests that the settled dust could be used for monitoring occupational exposure to fungal allergens in farm environments. Most floor dust samples from poultry farms had low Alt a 1 levels (median = 0.37 µg/g) but there was a broad range of concentrations (Fig. 2). In our study, we sampled the poultry farms in the early spring when the outdoor *Alternaria* spores are less prevalent. Therefore, our results suggest that the majority of dust-borne Alt a 1 in poultry farms may originate from the *A. alternata* accumulated in indoor dust, rather than from outdoor-indoor transport. Several studies reported high airborne spore counts of several allergenic fungal species in various buildings used to house animals, and higher concentrations of total fungi have been found in poultry farms than in buildings used with other animals 18 35-39. However, to our knowledge, no information is available regarding the indoor Alt a1 level in different animal housing buildings. Only a few studies have investigated Alt a 1 level in house dust samples by using the same mAbs ELISA 27 40. The frequency of detection of allergen Alt a 1 in the house dust samples is very low compared to that found with other indoor allergens (dust mites, cockroach, dog, cat). In Spain, Alt a 1 was detected most frequently in samples from storerooms, and the amounts of Alt a 1 were low (median = 0.37 µg/g) 40. Therefore, the routine application of this immunoassay has not been suggested for quantification of house dust-borne Alt a 1 41.

Results of this work show that the floor dust samples collected in poultry farms may not just act as the reservoirs of mite allergens 42, but also be a source of the fungal allergen Alt a 1. Studies with the dust mite allergens suggest that the exposure to 2 µg of allergen per gram of dust is associated with sensitization, and to 10 µg with respiratory symptoms in already sensitized persons 43 44. A recent study by Salo *et al.* 3 proposed a cutoff point of 7 µg/g for overall *A. alternata* antigen exposure in house dust samples. The interpretation of fungal exposure is complex, and no standardized method for assessment of fungal allergen exposure exists as yet. Therefore, the exposure threshold for fungal allergens has not been internationally accepted 2. Several epidemiological studies have indicated a

greater risk of respiratory disorders in farmers than in the general population [20](#) [22](#). Furthermore, a long-term exposure to even low fungal spore counts (and presumably to Alt a 1) may pose a risk of respiratory diseases [17](#) [45](#). Thus, the importance of fungal allergen measurements in farming environments is of great concern in a view of potential respiratory health effects due to high occupational exposure to bioaerosols including fungi.

Dust can be a carrier and source of nutrients for fungi, especially for dominant species such as *Alternaria*, *Aspergillus*, and *Penicillium* spp. [39](#). Settled dust shows less variability than the single-time-point air sampling for aeroallergens, and is generally accepted for assessing the residential levels of indoor allergens, particularly in epidemiologic studies. As we have sampled the poultry farms in the early spring, we expect that Alt a 1 level during the peak summer season of *Alternaria* would be higher. Consequently, repeated measurements through the year are required for an accurate assessment of annual Alt a 1 levels in poultry farms and housing facilities for other animals [14](#).

Exposure to organic dust, including fungal allergens, may vary qualitatively and quantitatively from one occupational setting to another [17](#) [45](#). Wood-related workplaces are also associated with significant exposure to fungi [46](#) [47](#). We found no detectable Alt a 1 levels in any of 15 dust samples ($< 0.12 \mu\text{g/g}$, detection limit) collected in one sawmill located near Zagreb (Croatia). These results are in agreement with Vailes *et al.* [27](#) who reported low detectability of Alt a 1 in the house dust samples using the same method as was mentioned previously. However, the literature indicates that *Aspergillus fumigatus* is the dominant agent of some allergic diseases in the wood processing industry [17](#). Therefore, future studies assessing occupational allergen exposure in wood-related workplaces should focus on *Aspergillus fumigatus*.

Our results show that described capture ELISA using mAbs is a very sensitive, specific, and reproducible method for quantification of Alt a 1 in dust samples collected in poultry farms. The detection limit of $0.12 \mu\text{g/g}$ was similar to the results reported elsewhere [27](#). The limitations of this survey is a relatively small number of dust samples collected from the poultry farms and in the sawmill. To our knowledge, this is the first report describing Alt a 1 levels in these occupational settings.

In conclusion, our results show that settled dust can be a carrier of Alt a 1 allergen in poultry farms and

that the mAbs ELISA could serve as a method for its monitoring. Exposure to Alt a 1 should be taken into account together with mite allergens when assessing the occupational allergen burden and its effects on the respiratory health of farmers. Determination of other allergens (Asp f 1) from *Aspergillus* spp. might be included in future studies for fungal allergen exposure assessment. Considering the seasonal variation of *Alternaria* allergen levels, further research should focus on determining the indoor Alt a 1 in the summer.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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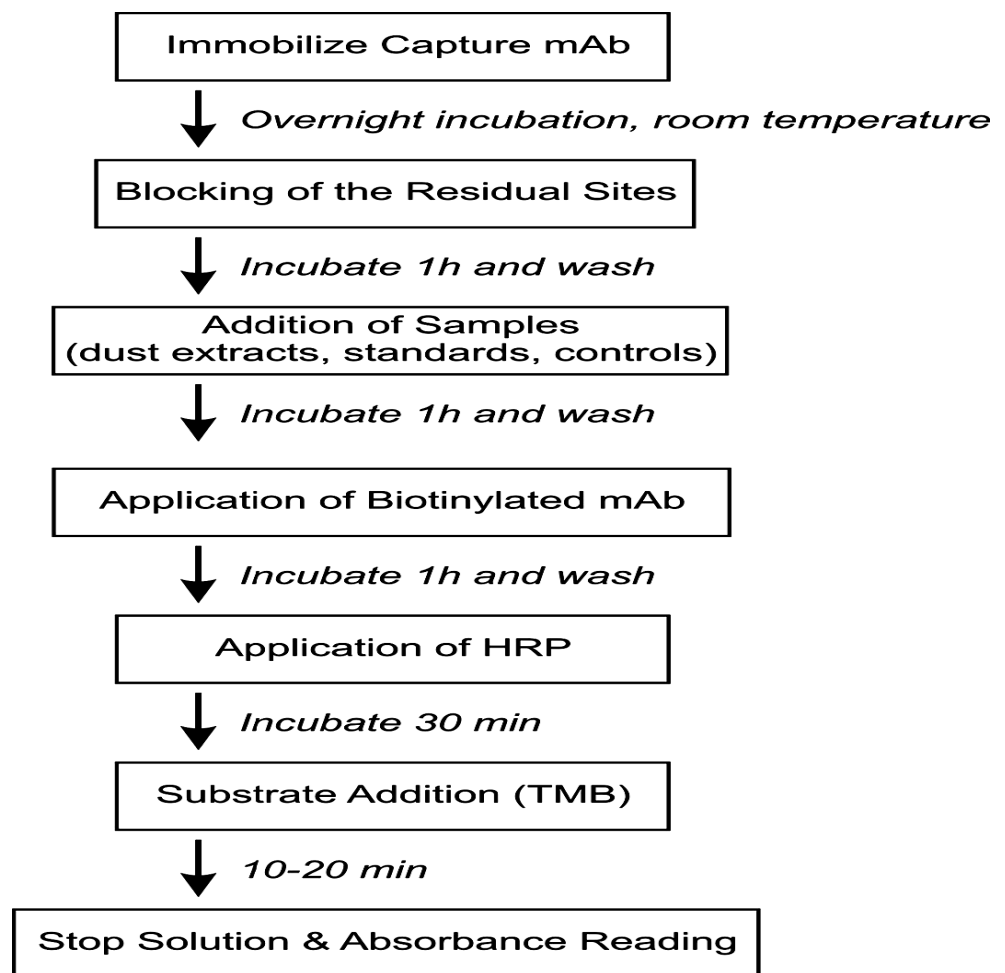
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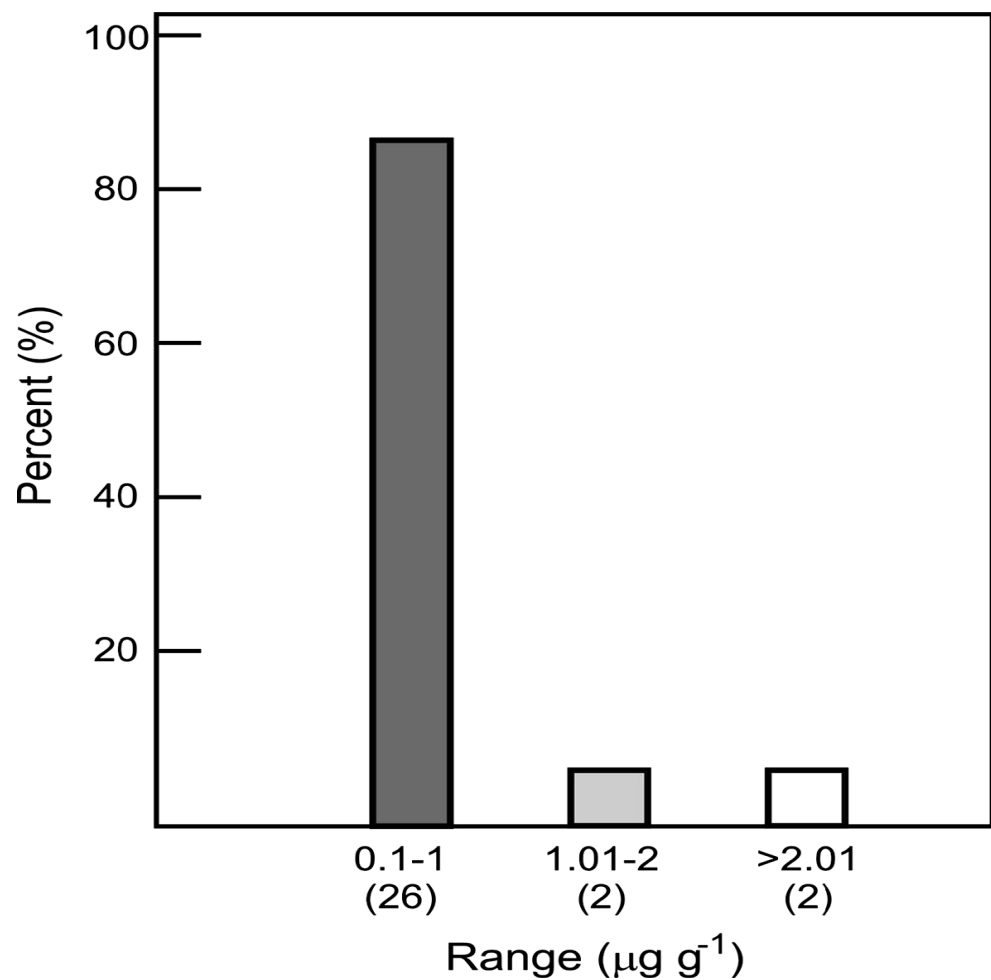
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[\[Enlarge Image\]](#)

Fig. 1. Flow diagram showing capture ELISA assay for determination of Alt a 1 in dust samples.



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Fig. 2. The percentage (%) of dust samples ($N=30$) from poultry farms where the Alt a 1 concentrations ranged in three levels: 0.1-1 µg/g, 1.01-2 µg/g, and > 2.01 µg/g. The number of participants in each group is indicated in parentheses along the abscissa.

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Table 1 Median, percentile and range of Alt a 1 (µg/g) in dust samples (n=30) from three poultry farms near Zagreb

	Median	25 th	75 th	Range	<i>n</i>
Farm 1	0.45*	0.24	0.84	0.14-14	18
Farm 2	0.33*	0.18	0.48	0.12-0.71	7
Farm 3	0.29*	0.24	0.59	0.15-0.61	5

*($P>0.05$).

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