globose monosulcate pollen with irregular columellar structure and a continuous tectum was ancestral, and regular columellar structure and a foveolate-reticulate tectum arose soon after. The oldest recognized Cretaceous angiosperm pollen may represent this grade of evolution. Granular structure was independently derived from columellar within Nymphaeales, Magnoliales, and Laurales, as well as in "higher" eudicots such as Fagales (Amentiferae). This agrees with developmental evidence that granular structure in Nymphaeales is a modification of columellar structure. These results refute one of the arguments for a relationship between angiosperms and Gnetales, Bennettitales, and *Pentoxylon* (the anthophyte hypothesis), namely the granular exine structure of these groups. Conversely, they remove obstacles to a relationship with "seed ferns" with alveolar exine structure (e.g., *Caytonia*, glossopterids) and/or Triassic Crinopolles pollen (with reticulate-columellar structure and thick endexine), which seemed less likely when granular structure was assumed to be primitive.

Magnoliales have been traditionally considered primitive but now appear to be a more derived early line. In Myristicaceae, members with granular exine structure are nested among columellar taxa and thus appear to be derived. Myristicaceae and Magnoliaceae (which are also columellar) are basal to *Degeneria*, *Galbulimima*, *Eupomatia*, and Annonaceae, which are united by a shift to granular structure. Within Annonaceae, granular monosulcate pollen (as in *Anaxagorea*) is ancestral, but it gave rise to columellar monosulcate and disulculate single grains and inaperturate tetrads, some of which underwent reversals to single inaperturate grains. The reticulate monoporate tetrads of Winteraceae were derived from foveolate-reticulate monosulcate pollen of the type seen in their sister group, Canellaceae. In Laurales, after an early shift to inaperturate pollen, the exine underwent stepwise reduction and granularization, culminating in the fragile exines of Lauraceae.

Session c1

BASIC AEROBIOLOGY/ MONITORING/ NEW TECHNIQUES: POLLEN

The use of a "solid phase cytometer" for a monitoring application to pollens and molds counts

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The solid phase cytometer (Chemscan system) is an ultrasensitive microbial analyser, designed to date and count microbial cells on a solid surface. The sensibility is sufficient to detect one cell. The solid surface may be a filter membrane or a glass slide.

Cells are labelled with fluorescent markers and analysed with a laser scanning system using a sophisticated discrimination process that can discriminate labelled cells from background environment such as autofluorescence particles. The process of cell labelling and laser scanning can be completed within one hour of sample collection.

This method is already validated for monitoring of process water in pharmaceutical industry and more recently for the detection of the airborne microorganism.

For monitoring pollen and molds, we work in three steps :

- Examination of the autofluorescence of pollens. That permit to count the total number of pollen of the sample in a few minutes. The sensibility and the reproducibility are very good.
- Production of specific rabitt antibody for the main allergenic or not particles that we want to study: betula, dactilys, parietaria, urtica, alternaria. These antibodies are controlled and conjugated with FITC (fluorescein isothiocyanate conjugated)
- Specific analysis of each kind of particles to study their own spectrometry (red and green).

Then mixing two and three kind of particles we can discriminate each specific particles and as they are fixed on a membrane it is possible to count them with a very high precision (one grain).

The last trials must help us to discriminate urtica and parietaria.

May the definition of Pollen Season influence aerobiological results?

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Pollen Season, Period of Maximum Pollen Production, Pollination Season, Pollination Period, Main Pollination Period, Main Pollen Season, Principal Pollination Period, Effective Pollen Season, Atmospheric Pollen Season, are amongst the terms most frequently used in the aerobiological literature to define the period of time in which most of the annual total pollen concentration is recorded. Similarly, different criteria are used to delimit this period, depending on the author, the pollen type and geographical area involved. The period is most commonly defined as the time of the year in which a certain percentage of the annual total pollen is recorded, the most frequent values used being 90%, 95% or 98% (Nilsson and Persson 1981, Torben 1991, Galán et al. 1995). In other cases, the period is defined in terms of the time elapsing between two days on which specific values or conditions are registered (Mullenders et al. 1972, Lejoly Gabriel & Leuschner 1983, Pathirane, 1975, etc.).

Onset of the pollen season can also be identified using different criteria: amongst others, the day when the daily pollen concentration reaches a given percentage of the annual total, the day when a certain daily pollen concentration value is achieved or when prime pollen grains are registered on a continuous basis.

In order to determine whether the definition of Pollen Season influences aerobiological results, pollen data were selected for four pollen types from three different geographical areas (Ourense and Córdoba –Spain- and Bologna –Italy-) recorded from 2001 to 2003. The selected pollen types were monospecific (*Betula, Platanus* and *Olea*) or plurispecific (Poaceae) and recorded very different annual totals in the different areas. The years chosen displayed different meteorological conditions and the three areas differed clearly in terms of biogeography and climate.

The onset of the pollen season was determined using over twenty different criteria, and a comparison of results obtained between pollen types and years in the same area and between areas and pollen type in the same year was made. Results were also compared with phenological results from the same areas.

The differences observed with respect to the onset of the pollen season as defined by different criteria varied depending on the pollen type. *Platanus* was, every year, the pollen type showing least difference in each area, while Poaceae displayed the greatest difference. The maximum difference was observed in Poaceae in Ourense, where a difference of more than 140 days in the onset of pollen season was detected in 2002 depending on the criterion used. Considerable differences between years were also observed for pollen types in all areas.

Weather conditions, the nearness of the pollen source, the possibility of long-distance transport, and the number of species included in the pollen type, influence the onset and duration of the airborne pollen season and can also provoke a gap between flowering start and atmospheric pollen season.

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LEJOLY-GABRIEL, M. & LEUSCHNER, R.M. (1983). Comparison of airborne pollen at Louvain-la-Neuve (Belgium) and Basel (Switzerland) during 1979-1980. **Grana**, 22: 59-64.

NILSSON S.; PERSOON S. 1981. Tree pollen spectra in the Stockholm region (Sweden) 1973- **Grana**, 20: 179-182. 1981

PATHIRANE, L. (1975). Graphical determination of the main pollination season. **Pollen et Spores**, 17: 609-610.

New techniques of capture and identification of aeroallergens

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Atmospheric aerobiological surveillance is a fundamental control tool for pollinosis, a very common, persistent and annoying illness. Pollinosis affects a large number of people and constitutes an important public health problem. Clinical and epidemiological studies are carried out to determine allergic cause-effect symptoms associated to pollen grains. In the clinical settings the skin-prick test is conducted, but other tests are also in use, such as the conjunctival, bronchial and nasal provocation tests with extracts of pollen allergen. Epidemiological studies have compared some measures of asthma symptoms with pollen count in the lower atmosphere, through optic microscopy count and recognition of collected pollen samples. Most of pollen allergens diffuse out the wall when the pollen grains were placed in the water or wet medium. The same or similar biochemical processes may be occurring in the sensitised patients mucosa and can induce allergic reactions. This mechanism may be responsible for symptoms of allergic rhinoconjunctivitis but cannot explain the occurrence of allergic asthma during or after the pollination period (Grote et al. 2003). Pollen allergens can occur in airborne particles smaller than pollen, micronic and submicronic atmospheric fractions. Some authors consider these particles responsible of allergic asthma, because intact pollen grains are unlikely to intrude into the deeper airways because their size (D'Amato, 2001). For that reason, some authors stated that traditional pollen reports and forecast based on counts

should be replaced by, or at least, supplemented with allergen reports (Rantio-Lehtimäki *et al.*, 1994, Beggs, 1998).

The aim of the present study is to compare the results of traditional pollen count vs. protein quantification for *Olea* and Urticaceae types. For traditional pollen count we used the well known Hirst method sampler VPPS 2000 developed by Lanzoni and for protein quantification the Burkard Cyclone sampler where respectively used. The Burkard Cyclone sampler is a continuous volumetric sampler with wind orientation using a single reverse flow miniature Cyclone with an air flow throughout of 16.5 l/min. The instrument offers 100% efficiency sampling down to the 1.06 µm range. Particles are collected directly into a 1.5 ml Eppendorf vial where they can be transferred and analyzed by immunological techniques. Target proteins for those pollen types are major allergens Ole e 1 and Par j 1-2. Comparison of pollen counts and aeroallergen quantification shows a good correlation with 99% significance (R *Olea*/Ole e 1= 0.9, R Urticaceae/Par j 1-2=0.7).

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Airborne pollen grain concentrations inside and outside homes in the Fresno (CA) Asthmatic Children's Environment Study (FACES)

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Little is known about the pollen grain concentration in indoor air. The composition and relationship of pollen concentrations inside and outside of homes, for neighborhood and regional distributions are poorly understood. This investigation of indoor and outdoor concentrations of pollen grains is a component of the Fresno Asthmatic Children's Environmental Study (FACES), which is designed to characterize the health effects of air pollution on asthmatic children living in Fresno County of California, USA.

From March 2002 to February 2003, 498 daily pairs of indoor/outdoor samples were collected at 83 homes. During the one-year period, 18 of the residences were sampled during two different seasons. The samples were collected with the Burkard Continuous Recording Air Sampler (Model 9100) continuously for 24 hours in the family room and outside each home at the height of 1.5 m on 5 days during a two-week period. Homes were selected by geographic location and household characteristics. Simultaneous sampling of pollen grains was conducted with three Hirst-type Burkard Seven-day Recording Spore traps, one at 11 meters height on the U.S. Environmental Protection Agency's Supersite monitoring station located in central Fresno, and two others (at 4.5 meters height each) on trailers located at different school yards in the Fresno area.

All pollen grains detected indoors originated in outdoor sources. The indoor concentrations during the peak pollen seasons (March-May and September) could be > 25% of the outdoor concentrations (average 2 hr concentration > 50 pollen grains m^{-3} air). The higher pollen numbers were strongly correlated with the time the windows were open. The highest indoor pollen concentrations were generally measured during the afternoon and night hours. *Morus* spp., Poaceae, *Quercus* spp. and *Pistachia* spp. were the most frequently found pollen in the spring season, *Ambrosia* and *Ulmus* spp. (*Ulmus chinensis*) in September. If the windows were closed, the indoor pollen concentration was very low or no pollen was detected. The presence of cats or dogs did not increase the indoor pollen concentration but in general the homes with pets had open windows for at least a part of each day. The spatial and seasonal variation of the pollen concentration was well demonstrated in the sampling.

The total pollen grain concentrations at the 4.5 m height were slightly greater than at 11 m sampling height, whereas the sampling at 1.5 m height often resulted in 10 times greater pollen concentrations than at the 11 m sampling level. The counts of *Morus* spp. , Poaceae and the autumn flowering *Ulmus* spp. were especially high. There was no *Ambrosia* spp. pollen detected at the 11 m sampling height during March and the concentrations in the autumn were low. At the 1.5 m sampling height *Ambrosia* spp. occurred in both seasons and in September the counts were 3-4 times greater than at 11 m sampling height. Outside the investigated homes, at the 1.5 m sampling height, the night time concentration of Poaceae during the main grass pollen season was generally twice the morning (6am – 12noon) concentration and nearly three times greater than the afternoon (12noon-8pm) concentration. For all species at the 11 m sampling height the start of the pollen season was delayed by 1-2 days, the peak diurnal concentration was delayed by 2-4 hours, and the pollen seasons were generally 1 week longer in duration compared with the 1.5 m sampling height.

The results show the importance of continuous sampling, and fine time resolution for analysis (2hr) of pollen grains in health related environmental studies.

Pollen-Project "From atmosphere to allergies". Diffusion models of pollen aerobiological bulletin in the plain of Bologna (Northern Italy)

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The Plain Monitoring Station of San Giovanni in Persiceto (near Bologna, Northern Italy; (17 m a.s.l.) has been running since the year 2000 with the aim of carrying out aerobiological monitoring on an open plain environment, which is characterised by a vegetation quite different from urban areas. San Giovanni is a little town, about 30 km from Bologna, Ferrara and Modena, characterised by a quite completely rural environment, and with a rapidly expanding handcraft economy. Many public and private organizations are joined in the Pollen-Project "From Atmosphere to Allergies" which involves the monitoring station of San Giovanni: our Laboratory of Palynology, University of Bologna, Italian National Research Council (ISAC-CNR, with the group of Prof. Paolo Mandrioli), two health institutions (ARPA-SMR and AUSL) and fifteen towns of the Province of Bologna, the Province itself and some local sponsors. The station, that is active all the year, records direct data about air quality, and its biological and abiological components, first of all in order to give valuable and timely information to doctors (municipal doctors, paediatricians), specialists in pulmonology and allergies and patients suffering of allergy for hay-fever prevention and treatment.

In order to obtain this goal, we proposed the set-up of two allergenic pollen bulletins: a) one model was assessed for specialists (doctors, paediatricians, specialists in pulmonology and allergies, pharmacists), and it is directly sent to hospitals, surgeries, medical centres, social welfare centres, pharmacies; b) the other model, more popular, was assessed for citizens, and it is sent to schools, social centres, libraries, consulting rooms halls, municipal offices of the province of Bologna, and directly to people suffering from allergy. According to standards of the Italian Aeroallergen Network, this second model quotes a colour for each family indicating weekly concentration values (white = lack of pollen in the air, green = low presence, yellow = medium presence, and red = high presence). The popular bulletin is weekly displayed in special show-cases in municipal Public Relation Offices, and in other "strategic" points of municipal areas, in consulting room halls, schools, and also in chemistries. The diffusion of the two types of bulletins is made by mail, fax, e-mail and web-sites. The models of pollen bulletin here proposed, even if open to any improving, are well suitable for easy and dispersal of information to specialists and the general public. The project is in progress, and other monitoring stations will be added (for example, by 2003 the San Pietro Capofiume station is also running) in order to find any differences about pollen quantity and quality. The area of diffusion of these bulletins will be improved by sending to more municipalities choosing those which are nearby or having the same environmental features of San Giovanni (small and medium sized towns, and in general plain areas of the region).

Estimations of feature space parameters for aerobiological analysis of pollen grains

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Image analysis is one of the approaches to the problem of automated identification schemes for pollen grains that offers advantages as an alternative to visual microscopic pollen counting by an expert. Image recognition techniques involve digital measurement of features for each pollen grain, including morphological features, surface features and other applicable characteristics. Conventional human inspection is still a difficult and time-consuming task even if it considered as a self-learning process supported by experts. Automated object recognition provides reproducible data with known quality and faster availability of the data.

The goal of this study is the development of the feature space parameters for pollen grain recognition and classification. The image analysis techniques autonomously extract all required information for the recognition from a data base constructed of acquired images. For this purpose it is necessary to calculate very specific features of all characteristics that can be useful to achieve differentiation in the routine recognition process for classification of different pollen grain types during months of high pollen counts.

The isolated pollen grains extracted from microscopic images are the objects digitized with high color standard. The strong conditions for further analysis are assumed due to variation of coloration used during preparation of the slides from the Burkard trap and sensitivity of some airborne pollens to the colorant. Segmentation algorithms specially tailored to pollen-object characteristics provide exact descriptions of pollen boundary and internal features (cytoplasm, pores, reticulum, etc) as used also by a human identifier.

The specific characteristics and its measures are statistically estimated for each object. For this stage of analysis statistic-based techniques are used for segmentation by means of with-class, between-class and total measures of the objects. Some low-level statistics for estimated local and global measures of the features establish the so called feature space. Estimated features with different measures describe morphological information as shape, color, central and invariant moments, texture and others.

Some special care should be paid in choosing these features to be described by estimated measures and on constructing the feature space. For each object the feature vector is calculated based on estimated measures during the description process in segmentation procedure. Covariance analysis with the measures of similarity between a query case and the class prototype are used to optimize a classifier. The class prototype characteristics are calculated taking into account vector of each class in the training set as in standard knowledge-based techniques.

The key factor is the certain choice of the pollen object characteristics, their specific measures, the number and the statistic level of their estimators. The goal of optimizing the number of subspace characteristics for feature vector is achieved by tailored low dimensional classifiers based on Nearest Neighbor algorithm with Mahalanobis distance measures.

The results of analysis for estimated parameters of the feature vectors in low dimensional space for some typical pollen types, like Alder, Hazel, Birch, Beech and Hornbeam, are presented as well as some results for performed experiments of effective and fast recognition for other pollens. The findings show the evidence of using proper chosen estimators of certain tailored characteristics for high recognition rates in classification even for low dimensional sequentional classifier for type differentiation of pollens grain.

Pyrogallol red, a new stain in aerobiology

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Pyrogallol red (aroprot®) was introduced as staining solution for determination of proteins and glycoproteins in aerobiology, being suitable for analysis of aerobiological samples of pollen, fungal spores and

other biogenic particles. Pyrogallol red sodium molybdate buffer is commonly used for the quantitative determination of total protein in human urine or cerebrospinal fluid, using manual or automated procedures. This test is based on the procedure developed by WATANABE *et al.* (1986) which is a dye-binding colorimetric method utilizing a pyrogallol red-molybdate complex modified to equalize the reactivity of albumin and γ-globulin, thus providing good precision and linearity. The pyrogallol red combined with molybdenum acid forms a red complex (max. absorbance at 467 nm). When this complex is combined with protein in acidic conditions, a blue-purple color develops (max. absorption at 598 nm). VÖLKER *et al.* (1996) reported on the application of a pyrogallol red-vanadium complex for protein staining in animal tissues and cell cultures.

After staining with a modified pyrogallol red-metal complex (0.02 % in 30 % isopropyl alcohol, pH 2.2, light-sensitive), microscopic observation results of different types of native pollen (*Betula, Corylus, Gramineae, Artemisia, Pinus, Taxus*) collected between 1998 and 2003 as well as material from 2003 pollen monitoring are presented. Furthermore, staining intensities of layers of the pollen exine, intine and cytoplasm are compared. The histochemical reaction of the dye (red-purple to shining blue) in the layers promise improved morphological differentiation due to contrast enhancement and allows visualisation and recognition of protein content and maybe allergenic activity.

For staining pollen, fuchsin or safranin are common dyes applied with glycerine jelly as embedding medium. As pyrogallol red is characteristically staining (glyco)proteins, as a consequence, even protein containing parts of pollen disrupted or damaged by soot and crystal particles (sand) or fungal spores can be visualised. Compared to conventional dyes, the visual differentiation between pollen layers results in images of higher contrast and improved display of pollen morphology, which is particularly valid for the boundary areas of the cytoplasm.

Lactic acid is a widely used mounting fluid in fungal microscopic examination, often used with a dye (cotton blue, aniline blue, trypan blue). For myxomycetes, ascomycetes, rusts and smuts Shear´s mounting fluid (potassium acetate) is commonly used. They all stain hyphae and hyaline fungal spores, but with pyrogallol red pigmented hyphal fragments (conidiophores) and spores are stained. Effects of glycerine jelly (Lanzoni), water activity and pH on colour spectra (ranging from purple to blue) and colour intensity are described.

The results of this work comfirm the use of pyrogallol red staining solution as a biological indicator in terms of (glyco)protein detection and as an innovative contribution to the identification of both pollen and fungal spores in aerobiological monitoring. In addition to its use in conventional pollen monitoring and detection, the dye allows to display a correlation of staining intensity with the protein content. While protein staining techniques are becoming faster, easier and more sensitive through chemiluminescence and (chemi)fluorescence, traditional reagents such as Coomassie blue and silver stains are still regularly used for certain applications. Among these, pyrogallol red has promising potential to contribute to the validation of the correlation between the allergenic activity and antigen-antibogy reactions, especially when image analysis of proteins on SDS gels or nitrocellulose membranes are applied. Furthermore, it may have the potential to value current allergenic acitivity directly from microscopic observation.

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Ragweed and mugwort pollen in Szczecin, Poland

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The aim of the study was to analyse the ragweed and mugwort pollination in Szczecin in the years 2000–2003. Measurement were performed by the volumetric and gravimetric method. Pollen seasons were defined as the periods of 90% of the total catch. Ragweed and mugwort pollen are known as potent aeroallergens, often noted to enter into cross reactions. In the four years studied, the lowest concentration of ragweed pollen was observed in 2000 and equalled a few pollen grains in 1 m^3 per 24 h. In 2001 the highest airborne concentration of 30 grains in 1 m^3 per 24 h was noted at the end of August. However, the annual pollen count of ragweed in 2002

was very high, three times higher than in 2001. The pollen season started in the second decade of August and lasted till the beginning of September. The highest airborne concentration of 100 grains in 1 m^3 per 24 h was noted at the beginning of September on a sunny day with strong wind. Also in 2003 a high pollen count of *Ambrosia* was recorded with the highest value on Sept. 8th equal 84 grains in 1 m^3 per 24 h. The pollination season of mugwort started in the third decade of July and lasted to the end of August in all the years studied. The highest pollen count of this taxon of 187 p/m^3 was noted on July 31st, 2002. In the other years the pollen count of *Artemisia* was lower and did not exceed 140 p/m^3. In sensitive persons the symptoms of pollinosis occur after some threshold pollen count value, which for ragweed is 13 p/m^3, and for mugwort - 12 p/m^3. Therefore, the greatest thread by *Artemisia* pollen allergens was noted in the end of July and in August and by *Ambrosia* in the end of August and the beginning of September. Analysis of aeroplankton from different Szczecin city's districts has shown that the highest exposure to ragweed pollen allergens occurs in the Majowe, which is related to the presence of numerous specimens of *Ambrosia* in that district. The pollen count of mugwort was more abundantly present in the district Żelechowa, in the area with villas and gardens or garden allotments than in the district Pomorzany characterised by closely built blocks. Statistically significant correlations have been found between the ragweed pollen count in the air and the maximum wind speed, air temperature and relative air humidity and between the mugwort pollen count in the and air temperature and relative air humidity. The pollen count of the taxa studied is determined by the weather conditions, especially by the wind speed and relative humidity, diversity of local flora and long distance transportation.

Session c2

BASIC AEROBIOLOGY/ MONITORING/ NEW TECHNIQUES: FUNGAL SPORES

The detection airborne plant pathogen spores using immunological assays

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Plant disease epidemics require three conditions to be fulfilled: there must be susceptible host tissue (the crop); the environmental conditions must be appropriate for the pathogen to infect; and there must be pathogen inoculum available. For many plant fungal diseases the inoculum is in the form of airborne spores. Thus, monitoring for the presence of disease causing spores could give useful information on the risk of disease developing in crops. Unfortunately, monitoring airborne disease inoculum using conventional methods for fungal spore detection is difficult. Methods based on microscopy or culturing are often slow, require skilled personnel and can be inaccurate. Recently there has been substantial interest in the development of plant disease diagnostic kits based on the use of serological methods. These methods rely on the production of monoclonal or polyclonal antibodies to detect epitopes specific to the target fungus. The technology used in such immunological assays is usually simpler to use and cheaper than methods based on PCR-assays. Such methods could be potentially exploited for use in detecting airborne plant pathogen inoculum (or other airborne biological particles).

In this paper I will review the potential use of immunological methods for detecting and quantifying airborne plant pathogen inoculum. Although immunological methods have been used to detect airborne allergens there have been few applications to plant pathogens. Immunofluorescence has been used to identify spores of *Botrytis cinerea* deposited on the trapping surface of a Burkard spore sampler. Spores were collected on a modified tape in a Burkard sampler exposed in an infected onion crop and labelled directly on the tape using a *Botrytis*-specific monoclonal antibody. Immunofluorescence has also been used to detect *Mycospherella brassicicola* ascospores on Burkard spore trap tapes coated with bovine serum albumin as a support medium and blocking agent. A prototype rotating-arm sampler has been designed for use with ELISA (enzyme linked immunosorbant assay) to detect and enumerate airborne spores. The trap has been tested in wind tunnel experiments using *Alternaria brassicae* conidia and a monoclonal antibody against germinating conidia of *A. brassicae*. A new particle-trapping device, the MicroTiter ImmunoSpore Trap (MTIST), for the rapid detection of