(54) Title: ANTIGENIC COMPONENTS OF SELECTED INDICATOR FUNGI AND THEIR USE IN METHODS AND TEST KITS

(57) Abstract: The present invention is related to antigenic components which are specific for three indicator fungi, *Stachybotrys chartarum*, *Aspergillus versicolor* and *Mennoniaella echinata* and which do not cross-react with other fungi common in the environment. Also disclosed are their use for producing binding substances and manufacturing test kits for evaluating the quality and extent of fungal contamination, remedial measures needed and checking the success of applied remedial measures as well as their use for checking whether a person has been exposed to the indicator fungi and/or if there is a link between allergic response of a person and the exposure to the indicator fungi.
Antigenic Components of Selected Indicator Fungi and their use in Methods and Test Kits

The Technical Field of the Invention

The present invention is related to antigenic components specific for three toxigenic indicator fungi, Stachybotrys chartarum, Aspergillus versicolor and Memnoniella echinata and having substantially no cross-reactivity with other fungi. Also disclosed are their use for producing binding substances and manufacturing test kits for determining the presence or absence of said toxigenic fungi and for screening the quality and extent of fungal contamination, the remedial measures needed as well as for controlling whether the applied measures have been sufficient and effective. Also described is the use of the antigenic components for screening whether a person has been exposed to the selected indicator fungi and/or has a link between the exposure and allergic responses.

The Background of the Invention

Fungal growth in buildings is known to lead to production of air-borne particles allergenic as well as non-allergenic. People living or working in buildings with fungal contamination are characterized by reoccurring asthmatic responses as well as other repeating symptoms, e.g. irritation. Possibilities to detect, whether a fungal contaminated building is suitable or safe for the occupants would be highly desirable. The awareness of the relationship between environmental pollution, especially air pollution, and the increasing occurrence of allergies, especially asthma, has also created an increased demand to develop methods and tools for easy and rapid monitoring and screening to decide whether the allergenic challenge is caused by fungal contamination or whether it is caused by other irritating agents. The deleterious effects of contaminants should preferably be evaluated by estimating the presence and absence of certain indicator fungi, both before and after remedial measures. The methods and means should preferably be utilizable under field conditions, just on the site of contamination without complex devices and special expertise.

Due to the key role of mycotoxins, considerable efforts have been focused on developing methods for detecting certain toxin producing fungi, especially strains of Stachybotrys, Memnoniella, and A. versicolor in order to inhibit and/or eliminate their growth. The
evaluation of the effects of remedial measures requires efficient test-based follow-up studies. So far, a successful, rapid and reliable, on-the-site method and/or test kit allowing routine determination and evaluation of the quality and extent of fungal contamination has not been developed.

An easy on-the-site method would enable development of standards for evaluation of fungal contamination. The method and test kit would allow easy checking that the standards are followed. They would also enable evaluation of economical damages caused by improper conditions and would be advantageous for checking the effect of remedial measures by screening whether the applied remedial measures have been successful in removing hazardous or irritating fungal components.

Consequently, the practicing environmental consults, industrial hygienists, safety officers, health inspectors, veterinarians and/or other persons working with or being responsible for investigations and evaluations of possible health risks and the need of repair or other remedial measures to minimize fungal exposure in contaminated environments, encounter in their everyday work lots of problems for which an accurate, rapid, easily available on-the-site test kit is urgently needed. For said persons, it would be advantageous if one could accurately evaluate the quality, severity and extent of fungal contamination with an unbiased test system when a person presents with symptoms suggesting the presence of possible health hazardous fungal contamination. It would, especially, be advantageous to be able to differentiate between risky and less serious fungal contaminations. It would also be beneficial to follow up the effects of remedial measures. An unbiased test result indicating the severity of contamination also allows a more accurate prediction of the costs of the urgency and remedial measures required and the economic feasibility of the measures selected.

The above is a non-exhaustive list of some problematic situations frequently encountered by those working with and being responsible for contaminated buildings. A guiding confirmatory reply often would be urgently needed, but adequate simple solution to the problem is still lacking. The lack of methods and reasonably prized, easily available test kits, for making an effective, rapid, on-the-site assessment is a severe defect for the professionals in the field.

Conventionally, the diagnosis of fungal contamination in the environment has been based on visual examination of fungal growth, odour detection or analysis, cultivation of environmental samples on culture media, which selectively allow the growth of indicator
fungi or microscopic examination of environmental samples. However, the increased awareness that fungal contamination in buildings can be an important cause of health outbreaks, e.g. allergic reactions, has created an acute demand not only for therapeutic remedies, but also for diagnostic methods and means to evaluate the presence of hazardous indicator fungi and the quality and extent of contamination.

For years, cultivation methods have been used for detecting fungi in contaminated materials and in the air of problem buildings. However, because of the relatively long, generally at least one week lasting, incubation time for fungi, cultivation methods are often too slow, particularly in cases, wherein prompt decisions are needed for choosing the most appropriate remedial measures to remove contaminants from the building. In addition, only viable fungal particles can be detected with the cultivation methods and it is well-known that only 1 - 25 % of the airborne fungal spores are viable. In the case of Stachybotrys, only 10 % of spores present in the air have been assumed to be viable and detectable with the cultivation methods. Therefore, various chemical applications and direct counting microscopy techniques, such as determination of fungal biomass (1,3-β-glucan or ergosterol), extracellular polysaccharides, fungal metabolites (volatile organic metabolites or mycotoxins) and total (viable and non-viable) fungal particles, have been considered as alternative and faster methods for detecting fungal products. However, many of those methods, the development of which are still only in progress, are not sufficiently specific to recognize the most hazardous fungal species or will be too expensive for general use.

In practical situations, for example when remedial measures are planned in problem buildings, there is an urgent need to detect the presence of defined hazardous indicator fungi. This is true, especially in cases wherein contamination of Stachybotrys and other toxigenic fungi is suspected. Due to the broad substrate specificities and because there are no selective media for differentiating fungi, cultivation methods are not the methods of choice. Because chemical methods are directed to measuring fungal products, common to most fungi, the chemical applications are also too unspecific for indicating the presence of specific fungal genera or species. In addition, identification of fungal species with the cultivation or direct counting techniques requires special expertise and well equipped laboratory facilities, which are not always available. Correspondingly, efforts to identify mycotoxins from environmental samples are out of the question because of high prices, special expertise required and laboratory equipment needed for toxin analyses and certain insecurity in interpretation of the results and their meaning.
Thus, it is evident, based both on scientific knowledge and practical demand, that new methods are needed for detecting health detrimental fungal contamination, such as *Stachybotrys* or other indicator fungi, in different materials from contaminated environments. Immunochemical methods provide excellent alternatives, because they fulfill most of the prerequisites for the purpose. Immunochemical applications are generally selective and sensitive for the fungus concerned. They are also independent of the viability of the fungus. However, immunological methods such as Western blotting, ELISA and other methods require expensive instruments and well equipped laboratory facilities. Such immunochemical methods are relatively tedious and difficult to perform. Accordingly, they do not fully satisfy the need of a reliable, simple and rapid on-the-site test. Immunochemical test strips have been suggested as a solution to the problem in field situations. Test strips would provide sufficient accuracy for practical use to detect the presence of *Stachybotrys* or other selected indicator fungi in different samples from various surroundings. They would not require any expensive equipment or special expertise for use. In fact, some commercial products and/or tests based on immunochemical applications are available for certain mycotoxins, but so far no products have been developed, which could specifically detect and differentiate the defined indicator fungi from other fungi. Toxins are not the product of choice for determination because all *Stachybotrys*, *Memnoniella* or *A. versicolor* strains do not produce toxins or the toxin production may be initiated only by certain conditions, during fungal growth, e.g. in building constructions and because there is a very wide range of different toxic metabolites that those species are capable of synthesizing, all of which have not yet been precisely characterized, the detection of mycotoxins is not a suitable choice. Thus, the desired fungal product for determination should not be limited to stachybotryotoxins, but should enable the identification of the whole organism, e.g. a *Stachybotrys* species, instead of stachybotryotoxins.

Several fungal species are known to be allergenic and to cause allergic disorders. Fungal allergy mediated through immunoglobulin E (IgE) has been proved by the skin prick test or by detection of the antigen-specific IgE antibodies in serum samples. However, fungal species of the same genus have been reported to cross-react in various degrees and cross-reactivity has also been observed between species of different fungal genera. One reason for the cross-reactivity is that the extracts of different fungal species can contain same or similar allergenic components or is a result of unspecific binding of polysaccharides with antibodies. Despite the fact that fungal extracts are widely used in the clinical work and research, little attention has been paid to the characterization of cross-reactive components in fungal extracts. Cross-reactivity is a factor which has to be taken in ac-
count, when a fungal product to be determined is chosen for developing immunochemical on-the-site tests.

The methods, test strips or test sticks comprising the test kits of the present invention provide a solution to the above-identified problem by providing immunochemical methods and test kits, which are sufficiently specific, selective and rapid. The test kits are capable of specifically recognizing one or more antigenic components from defined potentially dangerous indicator fungi, either separately, one by one or in any combination, either consecutively or simultaneously, from the same sample source. The methods and test kits allow an effective, rapid assessment of the quality and extent of contamination and evaluation of possible health risks, need of remedial measures, etc.

Accordingly, the objectives of the present invention is to provide improved methods for detecting, controlling the presence of detrimental fungal growth and evaluating possible health risks caused by indicator fungi and thereby to prevent certain fungi from growing by applying appropriate remedies. Another objective of the present invention is to provide means for carrying out said method. The means comprise specific antigenic or allergenic fungal components originating from the defined indicator fungi as well as binding substances or other materials selectively recognizing said antigenic fungal components.

The Summary of the Invention

The present inventions provides a solution to the problems listed above by providing new methods for detecting fungal contamination of certain type. The method and the products and means utilized in said method are as defined in the claims of the present invention.

A Short Description of the Drawings

Figure 1 shows SDS-Page profiles of the S. chartarum (A), M. echinata (B) and A. versicolor (C) extracts.

Figure 2 shows the components of the S. chartarum (A), M. echinata (B) and A. versicolor extracts recognized by the S. chartarum -specific rabbit antisera.

Figure 3 depicts immunoblottings of different S. chartarum preparations
Lane 1. Untreated *S. chartarum* extract immunoblotted against *S. chartarum*-specific rabbit immune serum.

Lane 2. Periodate-treated *S. chartarum* extract immunoblotted against *S. chartarum*-specific rabbit immune serum. Removal of carbohydrates influenced antibody binding to the 18, 19, 32, 36 and 41 kDa components.

Lane 3. Untreated HPLC-purified 50 kDa component (arrow) of *S. chartarum* immunoblotted against a serum of a patient sensitized to *S. chartarum* in a moldy building.

Lane 4. Periodate-treated HPLC-purified 50 kDa component (arrow) of *S. chartarum* immunoblotted against a serum of a patient sensitized to *S. chartarum* in a moldy building. No change was observed in the antibody binding.

The Detailed Description of the Invention

Definitions
In the present invention, the terms used, have the meaning they generally have in the fields of human and veterinary medicine and diagnostics, especially in on-the-site diagnostics, mycology, toxicology, immunology as well as in immunochemistry. Some terms, however, are used with a somewhat deviating or broader meaning in this context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

The term "evaluating" means judging or assessing from recorded, measurable or visually detectable results, the quality and extent of fungal contamination and whether the remedial measures have been successful. The "evaluation" is based on the determination, measurement or detection of antigenic or allergenic components of certain indicator fungi using "binding substances" specifically recognizing and binding to said "antigenic components".

The term "environmental sample" means a subsample of material or dust from the environment suspected of being contaminated with fungi. Such subsamples are obtainable or collectable from building materials, constructions, accumulated dust, bedding materials, fodder, foodstuffs, etc. The subsamples should be representative, i.e. they should describe the damaged or contaminated area sufficiently well.

The term "specifically recognizing and binding" means that the substance used to detect the defined fungal contaminant, the so called indicator fungus, detects components specific for the indicator fungi, but not other fungal components which are common to
most fungi.

The term "indicator fungi" means certain toxigenic fungi with verified health detrimental effects, which have been selected for the present invention based on their health detrimental qualities and their capacity of producing toxins. The presence of "indicator fungi" indicates that remedial measures are urgently needed in order to avoid health risks. The most preferred "indicator fungi" selected for the purpose of the present invention are selected from the genera Stachybotrys, Aspergillus and Memnoniella and especially including the toxigenic species: Stachybotrys chartarum, Aspergillus versicolor and Memnoniella echinata.

The term "non-indicator fungi" means other fungi, which are ubiquitously present, but which are not toxigenic to the same extent as "the indicator fungi" of the present invention. They cannot be considered to cause an extreme health hazard. If "non-indicator fungi" were detected, they would cause false alarms, which is not desired in order to avoid unnecessary, generally very expensive special remedial measures and protective measures, while the remedial actions are carried out. The contamination with non-indicator fungi naturally leads to some remedial actions, but those actions are not extremely urgent and extensive. For example, they do not require special protection measures against exposure among repair men and occupants during remedial activities as in the case of contamination by toxigenic indicator fungi.

The term "cross-reactivity" means that an antibody is able to bind to an antigen that did not originally elicit production of said antibodies. Fungal species of the same genus have been reported to cross-react in various degrees. In addition, cross-reactivity has also been observed between species of different fungal genera. One reason for the cross-reactivity is that the extracts of different fungal species can contain same or similar allergenic or immunogenic components or unspecific binding occurs between the glycoproteins, polysaccharides and the antibodies.

The term "devoid of cross-reactivity" means that the selected antibody or binding substance elicited by the antigenic component or binding specifically to the antigenic compound does not recognize and bind to non-indicator fungi, but only recognizes the indicator fungi. In other words it is specific solely for the indicator fungi.
The term "selection" means that a certain component with desired properties, e.g. specificity and/or devoid of cross-reactivity is chosen by isolation from a large group of antigenic components, the properties, e.g. cross-reactivities of which have been compared with the properties or cross-reactivities of other fungi.

The term "manufacturing" means the production of "antigenic components" with desired properties by isolation, purification, characterization and selection by conventional methods and thereafter by per se known recombinant DNA techniques. The term also include the preparation of "binding substance" specifically recognizing and/or binding to said "antigenic components" and their use for preparing "binding substances" by conventional immunotechnical methods, hybridoma techniques and recombinant DNA techniques. Said "antigenic components" and "binding substances" can be used for production of the test kits in methods for determining the absence or presence of indicator fungi and assessing the quality and extent of the fungal contamination.

Preparations or compositions, comprising the components useful as "antigenic components" can be prepared by isolation and purification methods such as chromatography purification system, gel filtration chromatography, anion exchange chromatography, cation exchange chromatography, reversed phase chromatography, affinity chromatography, e.g. lectin columns, etc. The components obtained can be characterized by SDS-PAGE, Western blotting, etc. and the cross-reactivity can be tested by per se known methods.

The term "quality of fungal contamination" means that the fungal contamination is severe, e.g. because of a high probability that toxic components are present and said toxic components or contamination has a clear health risk causing impact on those exposed in the vicinity of the contamination. Generally, special and urgent remedial measures, such as extensive repair, sanitary and protective measures are required. The term "extent of fungal contamination" means the area contaminated with health detriment fungi and also indicates how widely spread or how large and grave the moisture and consequent fungal contamination damages are.

The term "diagnosing" means that the environmental consults, physicians, veterinarians, etc. decide based on results obtained with a specific immunoassay method or test kit if a person has been exposed to contamination of indicator fungi and/or if there is a link between the exposure and allergic responses. In such cases the presence of antibodies in serum samples obtained from the person suspected of fungal exposure is a confirmatory
result for exposure of indicator fungi, whereas the absence of antibodies means that no exposure is likely.

The term "direct means" includes such immunoassays in which agglutination of the antigenic components happens in presence of the corresponding antibodies. The agglutination can be recorded, either visually or spectrophotometrically, as the turbidity developed during the reaction. In other words it means that only antigen and antibody is required, whereas the term "indirect means" indicates that the binding substances, i.e. antibodies or the antigenic components are made detectable by providing them with suitable markers or labels, such as latex particles, metal alloys, liposomes, enzymes, fluorochromes, etc.

The term "antigenic components" means immunogenic components of fungal origin, which are capable of eliciting antibodies against said components. As a conclusion "antigenic components" in its broadest aspect in the present invention, covers not only allergenic components, but also components which are typical and/or specific for certain indicator fungi, preferably any antigenic fungal components which do not cross-react with antigenic components from other so called "non-indicator fungi". In the most preferred embodiment of the present invention the term "antigenic component" comprises components obtainable from antigenic preparations of Aspergillus versicolor, Stachybotrys chartarum and Memnoniella echinata, which have been shown to correlate well with the quality of fungal contamination.

The "antigenic components" and/or their amounts are determined with "binding substances" including binding proteins or peptides as well as antibodies, which specifically recognize the antigenic components of said indicator fungi. The term "binding substance, which recognize indicator fungi" in its broadest aspect means any substances capable of specifically recognizing and binding at least one, preferably more antigenic components or at least a part thereof. Such substances are, for example, receptors or binding proteins or binding peptides, capable of specifically binding antigenic components and not cross-reacting with others. Above all binding substances mean antibodies capable of specifically recognizing one or more antigenic compositions of the three indicator fungi of the present invention, either separately or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments or derivatives thereof. The prerequisite being that the "binding substances" specifically recognize at least one of the above-defined antigenic components and does not cross-react with others.
Said "binding substances" can be developed using any of the "antigenic components" of the present invention, as well as their fragments, derivatives and complexes, the only prerequisite being that the "antigenic components" are capable of acting as "antigens", i.e. eliciting antibodies. Thus, the term "binding substances" refers to any compositions or materials capable of eliciting an antibody response specific to said antigenic components or compositions or materials containing them. Said antibodies are producible by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding molecules or compounds like specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks. The most recent techniques include development of synthetic polymer binding substances.

The term "immunoassay" refers to a ligand-binding assay that uses a specific antigen and/or antibody capable of binding to said antigen or analyte, i.e. to methods or procedures capable of detecting and/or measuring the "antigenic components" from the three indicator fungi, wherein the active and specific reagents include at least one antibody or part thereof capable of specifically binding said "antigenic components". Immunoassay principles can also be used in detecting antibodies against fungal antigens in patient sera. These antibodies are measured by their specific binding to the corresponding fungal antigenic components.

Depending upon the immunoassay used, especially in competitive immunoassays and/or quantitative assays, "antigenic components" of the present invention are used as optional reagents respective standards. Well known examples of immunoassays are radio-immunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks. Basic types of immunoassays include above all "sandwich assays", which are defined below.

The term "sandwich assay" refers to an assay in which one antibody (or antigen molecule) is used to capture the analyte of interest, while another (often with different specificity) is used for detection. "Sandwich assay" also refers to an immunoassay using
chemical or immunochemical binding of the analyte to a solid phase and the immunochemical binding of the analyte to a solid phase and the immunochemical binding of a second (labeled) reagent to the analyte. In other words, "sandwich assay" refers to an immunoassay using at least two antibodies or one antibody and one antigen capable of detecting or quantifying the amount of antibody or antigen in a sample. For example, two different antibodies capable of binding two different, non-overlapping (non-competitive) epitopes on the antigen can for example be used. Different types of "sandwich assays" exist as described below.

The term "Lateral Flow Technique" refers to an immunoassay using immunochromatographic principles. It is typical for the test that the sample or test solution, which is in liquid form moves along a test strip in contrast to the "Flow-Through Technique" in which the test solution is allowed to flow through a membrane in a test device.

The term "Flow Through Technique" refers to an immunoassay often based on the sandwich technique. The antigen containing sample or test solution is applied as a spot and is allowed to diffuse through a membrane in a device.

"On-the-site assay" means a test which can be performed in the field or in the actual place of contamination. It does not require transport of the samples and refers to tests or procedures performed without any laboratory facilities and without the need of qualified laboratory personnel. The term corresponds to the term "bed-side assay" used in hospitals and health care institutions. "On-the-site assay" means tests made by environmental consultants, industrial hygienists, health inspectors or other responsible professionals under field conditions. Also included are so called "self-tests", where the tests can be carried out by the those occupying the contaminated building. "On-the-site assays" are preferably performed on "solid carriers" like test strips. Various allergenic components can be detected simultaneously on the same strip or stick.

The General Description of the Invention

None of the conventional methods for diagnosing fungal contaminations are fully satisfactory for indicating the quality and extent of fungal contamination and do not allow differentiation between indicator fungi with specific health risks and less hazardous, common, fungi. Based on the fact that certain fungal peptides are more specific than others in evaluating the health detrimental properties of fungal contamination in buildings and that there is also some differences in specificity and selectivity when diagnosing different con-
taminations, the present inventors have developed methods and test kits for assessing the quality and extent of fungal contamination based on immunochemical determination of selected, specific antigenic components originating from the defined indicator fungi and which components do not show major cross-reactivity with other fungi.

The quality of fungal contamination and associated health risks have been connected with certain fungi. Especially, fungi of the genus Stachybotrys, e.g. S. chartarum, but also other fungi, such as Memnoniella echinata and Aspergillus versicolor produce under certain conditions several highly toxic metabolites (trichotheceines by Stachybotrys and Memnoniella as well as sterigmatocystin by A. versicolor). Intensive production of trichotheceines has been observed, particularly, on moist high-cellulose and low-nitrogen materials, e.g. on wallpaper, gypsum board, in hay and straw. Ingestion of contaminated feed is the main exposure route for mycotoxins in animals. Symptoms of animal toxicosis after exposure to either trichotheceines or A.versicolor toxins include, toxic irritation of eyes, skin, and mucous membranes followed by necrosis, changes in blood parameters, hemorrhages as well as teratogenic and carcinogenic effects in many organs (especially in kidney and liver), severe disorders of the immune system including teratogenic and carcinogenic effects depending on the toxin dose. For humans, handling of contaminated materials and inhalation of airborne toxins containing spores, are the most potential routes of exposure. Thus, the risk of exposure to toxins is essential among farmers and those occupying and/or repairing buildings with contamination of the toxigenic indicator fungi. Acute symptoms of human toxicosis comprise several toxic, irritative and nonspecific symptoms, such as dermatitis, cough, rhinitis, burning sensations in the mouth and nasal passages, sore throat, fever, headache, diarrhea, feebleness, and fatigue, possibly painful, severe pharyngitis, and a burning sensation in the nose, and bloody nasal exudates. The long-term health effects of toxicosis in humans exposed by inhalation are still unknown. Due to its health detrimental properties, Stachybotrys and A. versicolor have been named two of the most important indicator micro-organisms for moisture and/or health problems in buildings by indoor air experts. According to the present knowledge, especially Stachybotrys chartarum is probably the most dangerous fungus in buildings with moisture problems, indicating advanced moisture damages and serious health implications for occupants of the affected buildings. The recent investigations have shown that the growth of another fungus, Memnoniella echinata is associated with the presence of Stachybotrys chartarum in contaminated materials. Pure cultures of M.echinata has also been indicated to produce similar or same toxins as S. chartarum. Despite limited evidence on health risks caused by exposure to M.echinata
published, this fungus is a subject of intensive research and it will probably be included as one of the most hazardous fungi in the future.

For the reasons described above, the present inventors chose *Stachybotrys* as the key indicator fungi for their studies, but also the other fungi, especially of the genus *Mennioniella* as well as species *Aspergillus versicolor* are applicable as useful indicator fungi. It was shown that unspecified determination of breakdown and/or toxic products of the indicator fungi was not satisfactory especially because of their cross-reactivity with break-down products of other ubiquitously present but less health detrimental non-indicator fungi and certain insecurity related to mycotoxin analyses.

The cross-reactivity between the different fungal species may be due to the same or similar antigenic components, for example enzymes, that are shared by several fungi. Also, carbohydrate components of the glycoproteins may cause unspecific cross-reactivity (Brouwer, J. 1996, Int. Arch. Allergy Immunol. Vol. 110: 166-173; Nissen, D. et al. 1998, Ann. Allergy Asthma Immunol. Vol. 81: 247-255). To study the cross-reactivity of the key indicator fungus, *Stachybotrys chartarum* was grown in artificial malt extract broth and in cellulose broth that simulates its natural cellulose-rich growth media. The cross-reactivity between *Stachybotrys chartarum* and 10 fungal species (*Alternaria alternata*, *Aspergillus versicolor*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Mennioniella echinata*, *Penicillium brevicompactum*, *Phialophora rebens*, *Phoma sp.* and *Ulocladium botrytis*) was studied by the inhibition immunoblotting method.

Accordingly, the present inventors studied the cross-reactivity between the selected indicator fungi and other common fungi not included in the group of toxigenic indicator fungi as defined in the present invention (Table 1 and Table 2). Based on the results obtained, they identified specific antigenic components which do not significantly cross-react with other ubiquitous fungi. Based on said selected antigenic components the inventors have been able to produce specific binding substances, e.g. monoclonal antibodies, which are capable of identifying one or more specific antigenic components originating from one or more of the indicator fungi and capable of differentiating between different kinds of fungal contamination. Said antigenic components and their specific binding substances are not only capable of recording the absence or presence of certain indicator fungi but are optimal for determining the quality, severity and extent of fungal contamination. They also overcome the cross-reactivity problems discussed above.
In the studies extracts of *S. chartarum*, *A. versicolor* and *Memnoniella* were shown to contain several antigenic components.

All the fungal species studied showed cross-reactivity with *S. chartarum*. Some cross-reactivity could already be observed at the low concentrations (2 and 20μg/ml) of inhibiting preparations, while a high protein concentration of 2 mg/ml inhibited regularly the binding of the *S. chartarum*-specific rabbit antiserum to at least 50% of the components of *S. chartarum*. However, a clear difference in the protein concentrations needed to inhibit the binding of the *S. chartarum*-specific antibodies to the components of *S. chartarum* was observed between the other fungal extracts and the *S. chartarum* extract. Such components could be detected, to which the binding components of the antiserum was difficult to inhibit with the other fungal preparations. To get a fungal preparation with the protein concentration of 2 mg/ml, intensive concentration of the extract had to be performed. Intensive concentration of extract is not feasible in field conditions. Thus, such components are not the components of choice in the present invention.

The glycoproteins of *S. chartarum*-extract were obtained by Boehringer-Mannheim DIG Glycan Differentiation Kit. The most abundant carbohydrate in the *S. chartarum*-extract was mannose. The carbohydrate moiety was present in components with the molecular weight of 30 kD or higher. The significance of the carbohydrate content of *S. chartarum* glycoproteins for the antibody binding activity was demonstrated by periodate treatment of *S. chartarum*-extract (removing sugars), followed by immunoblotting with the *S. chartarum* specific rabbit antisera (Example 5). These results were compared to the immunoblotting results of native (no periodate treatment) *S. chartarum*-extract. These results showed that the removal of carbohydrate moieties from *S. chartarum* extract partially decreased the binding of *S. chartarum* specific rabbit immune serum to components in the extract, except for the 27 and 50 kDa components, which remains unchanged.

The preliminary studies were performed using polyclonal antibodies and ELISA-tests. The polyclonal antibodies against said allergenic or antigenic components were not specific enough, but when specific antigenic components without cross-reactivity had been selected, it was possible to produce specific binding substances. Monoclonal antibodies of the present invention were developed according to the original technique of Köhler and Milstein (Nature 256, 495, 1975), whose methods for producing monoclonal antibodies are herewith incorporated by reference. Similarly, monoclonal antibodies
recognizing other epitopes of the antigenic fungal component can be produced by those skilled in the art based on well-known laboratory book methods.

The antibodies produced can optionally be tagged with a label or marker molecule capable of making the presence or absence of the indicator fungi alone or in any combination recordable. Various labels, markers or tags, also called tracer when combined with the antibodies or respective antigens, are known and described in literature, laboratory handbooks and patent publications. Such labels or markers are, for example, coloured latex particles, fluorochromes, liposomes, metal colloids, etc. However, it is to be noted that it is not necessary to use such labels and markers. In turbidimetric assays for example, polyclonal antibodies as such are used and when they bind to the antigen the sample solution gets turbid. The turbidity is caused by antibody-antigen-aggregates forming during the reaction. Said aggregates can be detected visually.

Based on the results obtained in the present invention and the antigenic components selected and binding substances produced, it was possible to develop specific methods and test kits for assessing the presence or absence of the three indicator fungi based on their specific antigenic components which do not cross-react with antigenic components of other non-indicator fungi ubiquitously present in environmental samples.

The antigenic fungal components, which allow the specific differentiation of various defined contaminants and which are most suited and effective, for the desired, specific, diagnostic purpose, were selected. Said antigenic fungal components were used to develop test kits and methods targeted to diagnose the quality and extent of fungal contamination. The present invention provides a highly effective diagnostic tool for evaluating the need and type of remedial measures in cases of fungal contamination. In addition, the methods and test kits provide effective means for follow-up studies and for monitoring the success of the remedial measures. Ultimately, the test could be used for self diagnostics, especially by environmental consultants, industrial hygienists, house sellers, buyers and builders as well as their health contentious clients.

As addressed above, the presence of certain indicator fungi are directly related to the fungal contamination and should serve as a specific and sensitive biochemical indicator. Results from Western blots carried out from environmental samples suggest that the presence of certain antigenic components with antibodies, e.g. polyantibodies, can be used to confirm the presence of indicator fungi and to indicate the quality and extent of contamination with minimal false positive and negative results.
It is important to notice that the preferred test kits and methods of the present invention are developed to provide a qualitative test, but semiquantitative or even quantitative tests can be developed. The results can be recorded visually or by an instrument either directly or indirectly by adding a substrate capable of making the binding reaction recordable. The invention disclosed in the present patent specification provides a highly effective diagnostic tool for an accurate evaluation of the kind of fungal contamination and measures needed to correct the situation. The method and kits of the present invention above all provide alternative on-the-site diagnostic tools for recording the presence or absence of certain indicator fungi and for evaluating the quality of the contamination. In addition, the method and test kit provides an effective and practical tool for follow up studies of the efficacy of remedial measures.

Any immunochemical test methods can in principle be used for evaluating the quality of contamination as well as for longitudinal or latitudinal screening of the effects of remedial measures. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid on-the-site tests. It is possible to prepare test sticks or test strips, which contain several different antibodies in separate zones and with which it is possible to detect several allergenic components simultaneously.

In a specific embodiment of the present invention, the investigator carries out a test by placing an absorbing test strip or corresponding solid carrier directly in contact with a subsample obtained by removing a dry sample of material from the area suspected of being contaminated. The dry sample is transferred into a suitable liquid carrier, such as a buffer and is extracted to provide the subsample. The test strip is thereafter allowed to absorb liquid from the subsample extract, preferably for a standardized time. Finally, a dipstick-type test device can be so designed that it includes an absorbing end that is placed in contact with the sample source, which is absorbed directly into test device.


Recombinant allergens can also be used for determining fungus specific antibodies in serum samples of human exposed to the indicator fungus. E.g. elevated levels of specific
IgG indicate increased exposure to the indicator fungus and high levels of specific IgE refer to possible allergic responses against of the fungus. By using verified antigen components, the antibody measurements become more reliable and accurate compared to the present techniques.

As a conclusion the present invention is related to methods and test kits for determining the presence or absence of the defined indicator fungi or their antigenic components in environmental samples and for evaluating the quality and extent of fungal contamination in sick and moisture damaged buildings, houses, factories, agricultural environments and other working environments, for evaluating the need and urgency of remedial and protective measures to avoid health risks caused by fungal contamination, for targeting remedial measures to reduce and/or eliminate contamination and for evaluating whether the applied measures have been successful. Preferably, the diagnosis should be performable as a rapid and reliable on-the-site assay using one or more binding substances capable of specifically recognizing one or more specific antigenic components originating from the defined indicator fungi, e.g. *Stachybotrys* alone or in combination with some other indicator fungi in environmental samples.

The feasibility and applicability of the methods and test kits of the present invention are illustrated described in the following examples describing the experiments performed and their results. Even if the Example is restricted to the development of a kit based on antigen components of *S. chartarum* and its specific binding substances, it is evident, based on the preliminary cross-reactivity results of Table 1 & 2 that similar antigenic components and binding substances can be prepared for the two other indicator fungi of the present invention.

**Example 1.**

**Cultivation of the indicator fungi and the other fungi in malt extract broth**

The indicator fungi, *Stachybotrys chartarum* (strain ATCC 208877) was isolated from gypsum board derived from a moisture damaged building in 1991. The strain was identified at Centraalbureau voor Schimmelcultures (CBS), Baarn, the Netherlands. The other fungal species studied were *Alternaria alternata* (DSM 62006), *Aspergillus versicolor* (UKU 3), *Aureobasidium pullulans* (DSM 62074), *Chaetomium globosum* (UKU 4), *Cladosporium cladosporioides* (DSM 62121), *Memmniella echinata* (CBS 304.54), *Penicillium brevicompactum* (ATCC 58606), *Phialophora rebens* (KTL RE27), *Phoma sp.* (UKU 8), *Ulocladium botrytis* (UKU 11), *S. chartarum* (KTL 296), *S. chartarum* (KTL 391), *S. chartarum* (KTL 530)
and S. chartarum (KATTL). All the strains coded by UKU and KTL were identified at CBS and the others (except KATTL) were purchased from international fungal collections.

The fungal species were first allowed to grow on an artificial 2% Malt extract agar for 7-10 days at 25 °C, after which spore suspensions were prepared in phosphate-buffered saline (PBS) with 0.05% (v/v) Tween 20. One ml of the spore suspension was inoculated in 100 ml of 2% Malt extract broth in stationary Roux bottles and the cultures were incubated for 10 days at 25 °C.

**Example 2.**

**Cultivation of the indicator fungi and other fungi in cellulose broth**

Fungal species mentioned in Example 1 were allowed to grow as described in Example 1 in cellulose containing liquid broth (Dillon, H. K., Heinsohn, P. A., Miller, J. D. (eds), Field Guide for the Determination of Biological Contaminants in Environmental Samples. AIHA Publications, Fairfax, Virginia 1996) that simulates the natural growing media (straw, gypsum board) of S. chartarum.

**Example 3.**

**Preparation of the antigen extracts**

Fungal cultures containing mycelia and spores were dried in a vacuum dryer and resuspended at 1:20 (w/v) in phosphate-buffered saline (PBS, pH 8.0) with 0.02% (w/v) phenylmethyl sulfonyl fluoride and 0.02% (w/v) natrium azide. Mycelium suspensions were homogenized and extracted in a shaker for 16-18 h at 4 °C. After centrifugation (1000 g, 10 min and 43 000 g, 60 min at 4 °C), the supernatant was dialyzed (molecular weight cut-off 8 kD) against three changes of PBS (pH 7.4) at 4 °C for 24 h, sterile-filtered (0.2 μm) and lyophilized. Lyophilized extracts were stored at -70 °C and resuspended in water for use. The protein content of the extracts was determined by the dye-binding method of Bradford (Bradford, M. M., Anal. Biochem., 1976:248-254).

**Example 4.**

**Rabbit antiserum to fungal antigens**

Three female New Zealand White rabbits were immunized subcutaneously four times at two-week intervals with the extracts (1 mg protein per rabbit per immunization) of S. chartarum prepared from malt extract and cellulose broth as well as extract of A. versicolor. The terminal sera were collected two weeks after the last immunization and stored at -70 °C.
Example 5.
SDS-PAGE, immunoblotting and inhibition immunoblotting
Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer were carried out using the methods described by Laemmli, U. K. (Nature. Vol. 227, 680-685 (1970)) and Towbin, H., et al. (Proc Natl Acad Sci USA. Vol. 76, 4350-4354 (1979)). For the immunoblotting analysis, the nitrocellulose membranes with electrotransferred proteins of antigen extracts were cut to strips and blocked with gelatin-PBS. To characterize the immunogenic components of the fungal extract, the membrane strips were incubated overnight with pooled terminal rabbit antisera at a dilution of 1:5000 and thereafter with peroxidase-conjugated swine anti-rabbit immunoglobulins at a dilution of 1:5000 for 1 h. All the incubations were at room temperature. Strips were washed with PBS-Tween several times. The autoradiographic reaction was developed by ECL -Western blotting reagents and the immunologically reactive protein components were detected by autoradiography film. In the inhibition-immunoblotting analysis used in cross-reactivity testing, the *S. chartarum/A. versicolor*-specific antiserum was first incubated at 37 °C for 1 h with several dilutions of the other fungal extracts. Thereafter, the analyses were completed as described before.

Example 6.
Comparison of protein profiles and antigenic components of *S. chartarum* between extracts prepared from the cultures in malt extract and cellulose broth
In the SDS-PAGE analysis, the *S. chartarum* -extract prepared from the culture grown in cellulose broth revealed more protein components (13) than the extract prepared from the culture grown in malt extract broth (12). In addition, the extract prepared from malt extract broth contained non-immunogenic components with a small-molecular weight that caused intense smearing of the coomassie-stained SDS-PAGE gel. The extract prepared from cellulose broth revealed also more immunogenic components in the immunoblotting analysis (18) than the other extract (12). In the extract prepared from malt extract broth, the components of about 92, 57 and 25 kD gave the most intensive reactions, while the components of about 60, 50, and 31 reacted strongly with the rabbit antiserum in the extract prepared from cellulose broth. The results obtained and shown in Figure 1 indicated that a cellulose containing broth is better than a malt extract broth for preparing extracts for preparation of antigenic components according to the present invention.
In the SDS-PAGE-analysis, *M. echinata* extract prepared from fungi cultured in cellulose broth revealed 13 protein components, the components of about 48, 50 and 60 kD being almost abundant (Fig. 1). The rabbit antisera raised against *S. chartarum* cultured in cellulose broth recognized distinctly the components 150, 110, 60, 38, 37, 36 and 32 kD in the *M. echinata* extract (Fig 2).

*A. versicolor* extract prepared from fungi cultured in cellulose broth revealed 18 protein components of which the components of about 100, 98, 85, 55 and 43 kD were the most abundant (Fig. 1). The rabbit antisera raised against *A. versicolor* cultured in malt extract broth recognized distinctly the components of about 125, 110, 98, 82, 62, 55, 50, 43, 37, 36, 32 and 29 kD in the *A. versicolor* extract prepared from fungi cultured in cellulose extract.

Example 7.

**Cross-reactivity results - selecting suitable antigenic components**

All the fungal species studied showed cross-reactivity with *S. chartarum*. Some cross-reactivity (0%-36% disappearance of the components of *S. chartarum* in the inhibition immunoblotting analysis could already be seen at the low concentrations (2 and 20 µg/ml) of inhibiting preparations, while the protein concentration of 2 mg/ml inhibited the binding of the *S. chartarum-*specific rabbit antiserum to 64-100% of the components of *S. chartarum*. In other words, the visualization with autoradiography was prevented or inhibited when cross-reaction had occurred. However, a clear difference in the protein concentrations needed to inhibit the binding of the *S. chartarum-*specific antibodies to the components of *S. chartarum* was observed between the other fungal extracts and the *S. chartarum* extract. The lowest protein concentration (2 µg/ml) of the *S. chartarum* extract prepared from the culture grown in malt extract broth used for the inhibition prevented the visualization of 45%, and the extract prepared from the culture grown in cellulose broth 65% of its components. The fungal species that are morphologically closely related to *S. chartarum* (*A. alternata, M. echinata* and *U. botrytis*) cross-reacted more intensively with it than the other fungi. Antigenic components of *S. chartarum* grown in malt extract broth, to which binding of the *S. chartarum-*specific antibodies was easily inhibited by the other fungal preparations, were those of about 67, 31, 20 and 17 kD, while the binding was difficult to inhibit to components of about 77, 64, 57 and 25 kD. Binding of the *S. chartarum*-specific antibodies was easily inhibited to the components below 50 kD by the other fungal preparations, while the binding was difficult to inhibit to the components of about 60 and 50 kD. The five other strains of *S. chartarum* tested inhibited strongly the binding of the *S. chartarum*-specific rabbit antise-
rum to the components of *S. chartarum* in the immunoblotting analyses indicating similar antigenic components.

The rabbit antisera raised against *A. versicolor* cultured in malt extract broth recognized distinctly 11 components in the *S. chartarum* extract cultured in cellulose broth. In the *M. echinata* extract the figure was 5 components (110, 98, 82, 62 and 55 kDa). The rabbit antisera raised against *S. chartarum* cultured in cellulose broth recognized distinctly one component (60 kDa) in the *A. versicolor* extract cultured in cellulose broth (Fig. 2). In the *M. echinata* extract the figure was 6 components (150, 110, 60, 38, 37 and 32 kDa) (Fig. 2).

The components of about 60 and 50 kDa proved to be the most specific for *S. chartarum* cultured in cellulose broth in the inhibition-immunoblotting studies (Table 1). According to the inhibition-immunoblotting studies (Table 1), the 50 kDa component may be shared between *M. echinata* and *S. chartarum*, but the fact that the rabbit antisera raised against *S. chartarum* cultured in cellulose broth recognized only very faintly the component of 50 kDa in the *M. echinata* extract may refer to the fact that the cross-reactivity may possess an unspecific nature.

The components of about 85, 55, 35 and 33 kDa proved to be the most specific for *A. versicolor* cultured in malt extract broth in the inhibition-immunoblotting studies (Table 2).

**Example 8.**

**Characterization of antigenic components of *S. chartarum* grown on cellulose medium**

**Sugar analysis**

The glycoproteins of *Stachybotrys chartarum* -extract were determined by Boehringer-Mannheim DIG Glycan Differentiation Kit. The most abundant carbohydrate in the *S. chartarum*-extract was mannose. Other carbohydrates, for example galactose-β(1-4)-N-acetylglucosamine and sialic acid were also identified. The carbohydrate moiety was present in most components with the molecular weight of 30 kD or higher. The significance of the carbohydrate content of *S. chartarum* glycoproteins for the antibody binding activity was demonstrated by periodate treatment of *S. chartarum* -extract (removing sugars), followed by immunoblotting with the *S. chartarum* specific rabbit antisera (Example 5). These results showed that the removal of carbohydrate moieties from *S. chartarum*-extract partially decreased the binding of *S. chartarum*
-extract specific rabbit immune serum to the several components in the extract. The 50 kD component, previously shown to be specific for \textit{S. chartarum} remains unchanged. Also, after the periodate treatment, it was still recognized by the rabbit immune serum and by the sera of patients sensitized to \textit{S. chartarum} in moldy buildings. However, the component contained a weakly stained galactose-\(\beta(1-4)\)N-acetylglucosamine carbohydrate moiety, but the removal of carbohydrates did not affect the antibody binding of the 50 kD component.

At least one hour treatment with 50 mM Na-periodate at dark was needed to remove the sugar moiety from the mold-extract. The effectiveness of the periodate treatment was performed by further glycoprotein analysis with Boehringer-Mannheim DIG Glycan Differentiation Kit. Sugars (mannose, galactose-\(\beta(1-4)\)-N-acetylglucosamine and sialic acid) identified in native \textit{S. chartarum} extract could not be identified after periodate treatment.

N-glycosidase-enzyme F endoglycosidase-enzyme (Boehringer-Mannheim) was used for hydrolyzation of all types of N-glycan chains from glycoproteins (removing sugars). After treatment, the molecular size of the 50 kDa component remains unchanged, while most of the other components in the \textit{S. chartarum}-extract profile (SDS-page) were either disappeared or the molecular size was changed (reduced) as a sign of removed carbohydrate content.

\textbf{Example 9.}

\textbf{Characterization of antigenic components of \textit{S. chartarum} - chromatography purification methods}

1 \textbf{Gel filtration chromatography}

The frozen samples were re-suspended in PBS (pH 7.4) and dialyzed (molecular weight cut-off 8 kD) against three changes of PBS at 4°C. Max 250 \(\mu\)l samples of mold-extract were injected to the superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden). PBS (pH 7.4) was used as an eluent. All peaks were collected, starting from the first peak, equilibrated with the same buffer.

\textbf{Anion exchange chromatography}

The frozen samples were re-suspended in PBS (pH 7.6) and dialyzed (molecular weight cut-off 8 kD) against three changes of 20 mM Tris (pH 7.6) at 4°C. Max 2ml samples of mold-extract in 20 mM Tris (pH 7.6) were injected to the Resource Q HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The bounded
proteins were eluted with linear 90 ml gradient from 0 to 1 M NaCl in 20 mM Tris (pH 7.6) and all peaks were collected.

**Cation exchange chromatography**
The frozen samples were re-suspended samples in 20 mM citric acid (pH 2.7) and dialyzed (molecular weight cut-off 8 kD) against three changes of 20 mM citric acid at 4°C. Max 2ml samples of mold-extract in 20 mM citric acid (pH 2.7) were injected to the Resource S HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The bound proteins are eluted with a linear 90 ml gradient from 0 to 1 M NaCl in 20 mM citric acid (pH 2.7) and all peaks were collected.

**Identification of the isolated peaks by SDS-PAGE**
Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 15% acrylamide gel with the discontinuous buffer system of Laemmli (Laemmli, 1970). Briefly, a 50 μl sample from each fraction was reduced with 30 μl of the same buffer containing SDS and 2-mercaptoethanol, and the samples were boiled for 10 minutes. The samples and Pharmacia Low Molecular Weight calibration kit standards (Pharmacia Biotech) were loaded to the gels and the electrophoresis was carried out at 200 V for 60 min. The gels were stained with Coomassie brilliant blue.

**Immunoblotting of the isolated peaks transferred to a nitrocellulose filter**
Immunoblotting analysis were made as described in example 5.

**Example 10**
**Characterization of the isolated components**
**Isolation of the specific fragment from the SDS-PAGE, amino acid sequencing of proteins**
**N-terminal sequencing**
The proteins from SDS-PAGE gel were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 100V for 30 min. The membrane was stained with 0.1 % Coomassie brilliant blue and destained with 50 % methanol. Stained 50 kD band was excised with a scalpel and they were allowed to dry at room temperature. Applied Biosystems 477A Pulsed Liquid Phase protein sequencer was used to determine the N-terminal amino acid sequence of protein. No amino acid sequence was achieved since the N-terminal end of the protein was blocked.
Sequencing of internal peptides
In-gel protease digestion for amino acid sequencing of peptides was done with minor modifications as described by Rosenfeld et al. (Rosenfeld et al., 1992). SDS-PAGE was carried out as described above. The specific protein band of a gel was cut out and washed two times with 50% acetonitrile-ammonium hydrogen carbonate buffer (200 mM) at 37°C for 45 min. Subsequently the gel slices were shrinked with acetonitrile and then the samples were reduced with dithiotreitol (30 min at 56°C) and carboxymethylated with iodacetamide (15 min at room temperature at dark). Dried gel slices were swollen partly with 10 μl of 100 mM ammonium hydrogen carbonate buffer containing 10% acetonitrile and 0.5 μg TPCK treated trypsin (Sigma). 100 mM Tris-HCl buffer, pH 9.2, 10% acetonitrile was added slowly to cover the gel slices, and the incubation was carried out overnight. The peptides were extracted twice at 37°C in a shaker with 60% acetonitrile - 0.1% TFA. The digestion solutions were pooled together and dried to a about 50 μl volume. The generated peptides were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) (μRPC C2/C18 ST 4.6/100 column/ Amersham Pharmacia) with the gradient of 0.065% trifluoroacetic acid (TFA)/water - 0.05% TFA/ 84% acetonitrile. Applied Biosystems 477A Pulsed Liquid Phase protein sequencer was used to determine amino acid sequences of the peptides.

Example 11
Manufacturing antigenic components by recombinant DNA techniques. Isolation of messenger RNA and cDNA cloning
An isolate, strain UKU 10, S. chartarum was cultured on 6 x 10 ml liquid cellulose medium in stationary Roux bottles at 25°C. The mycelia was harvested on day 7. The mycelia was centrifuged (3000 x g, 10 min at 25 °C). The supernatant was discarded and the semi-dried mycelian mass was frozen with liquid nitrogen and stored at -70°C. The frozen extract was ground in a mortar and pestle under Qiagen RLC-reagent. The total RNA was isolated with Qiagen RNEnasy Plant Mini Kit-method. The messenger RNA was purified from the total RNA with Qiagen Oligotex mRNA Maxi Kit. A complementary DNA expression library was constructed with Stratagene ZAP-cDNA synthesis kit and cDNA was cloned into the Uni ZAP XR vector (Stratagene, La Jolla, CA).

Example 12.
Screening of the cDNA library
The cDNA library was immunoscreened using sera of patients with proved S. chartarum exposure histories and typical clinical symptoms of S. chartarum exposure or sensitization. The cDNA library was also immuno screened with the S. chartarum specific rabbit
anti-sera (Example 5). Phage plaques were lifted onto isopropylthio-β-D-galactoside-treated nitrocellulose filters (Amersham). Filters were washed, blocked by 0.1% casein in phosphate-buffered saline (PBS, pH 7.4) for four hours and further incubated with the 1:5 diluted human or 1:50 diluted rabbit anti-sera. HRP-labelled 1:10 diluted anti-human IgE (DAKO, P0295) and HRP-labelled 1:5000 diluted anti-rabbit Ig (DAKO, P0399) were used as secondary antibodies with a four hours incubation. Filter washings and serum and conjugate dilutions were made in PBS with 0.1% Tween. All incubations and washings were made at room temperature with gently agitation. The autoradiographic reaction was developed by ECL-Western blotting reagents (Amersham) and the immunologically reactive plaques were detected by autoradiography film (Amersham). Positive plaques were picked and purified with repeated phage dilutions and immunoblottings.

The amino acid sequence produced by sequencing of the internal peptides was used as a model for planning PCR-primers for cDNA library screening. Peptide M[NNVYFYGH (SEQ ID NO:1:) was used for producing degenerated PCR-primers. The other PCR-primers were planned for pBluescript SK(+/-) phagemid cloning vector. The upper strain primers were T3 primer AATTAACCCTCACTAAAGGG (SEQ ID NO:2:) and peptide based GTYCCNATYAAYATYCANCTY (SEQ ID NO:3:). The lower strain primers were T7 primer CGGGATATCAGTCACATATG (SEQ ID NO:4:) and peptide based GAYGTNAYTTYTYGNCAY (SEQ ID NO:5:). A is adenine, C is cytosine, G is guanine, T is thymine, Y means both C + T and N codes all A + C + G + T.

32P-labelled oligonucleotide probes were designed from the DNA sequence produced by PCR-amplification (described above) and cDNA library was screened according to the standard protocol of Sambrook, J. et al. (Molecular Cloning, A laboratory Manual. Cold Spring Harbor, New York, 1989)

Example 13
Sequencing of the cDNA clones
Inserts containing Bluescript SK(-) phagemids were excised from the bacteriophage Uni-ZAP XR lambda and transformed to Escherichia coli, XL1-Blue cells (Stratagene). DNA for sequencing was purified by Wizard Miniprep DNA Purification System (Promega). Nucleotide sequencing was done with the A.L.F. and A.L.F.Express DNA sequencer using Thermo Sequenase CY5 Dye Terminator Kit, 27-2682-01 (Amersham
Pharmacia Biotech). The DNA sequences were compared to the DNA sequences in Data-banks (NCBI GenBank) to identify the most specific *S. chartarum* DNA-fragments.

**Sequencing of the PCR-products**
PCR-products were isolated from agarose gel and purified by GELase™ Agarose Gel-Digesting Preparation method (Epicentre technologies) before DNA sequencing (described above).

**Example 14.**
**Production of recombinant proteins in *Pichia pastoris***

cDNA of *S. chartarum* (UKU 10) was used as a template for PCR. The primers were designed to include the leader sequence of specific *S. chartarum* protein as well as Eco R1 sites for cloning in the pHIL-D2 expression vector of *Pichia pastoris* (Invitrogen, Leek, the Netherlands). The other primers were designed for cloning in the pHIL-S1 expression vector of *Pichia pastoris*. The plasmid construction was performed with standard techniques (Invitrogen), and the correct insertion into the plasmid was verified by sequencing. The recombinant plasmid was transformed in *Pichia pastoris* strain GS115 (Invitrogen) by lithium chloride transformation method.

Seed cultures of transformed Pichia yeast were grown in a salt-based medium (BMG; Invitrogen) containing 1.0 % (v/v) glycerol on a rotating incubator at 30 °C. For the production of the recombinant protein, yeast cells were collected by centrifugation and resuspended in one-fifth of the same medium base without glycerol, methanol was added to 0.5 % (v/v) and the incubation continued for 6 days with daily adding of methanol. The supernatants of the cultures were harvested by centrifugation and the recombinant protein was purified with a procedure comprising affinity chromatography, ion exchange chromatography and gel filtration chromatography.

**Example 15:**
**Production of monoclonal antibodies against specific antigenic components of *S. chartarum***

**Antigen preparation**
*Stachybotrys chartarum* specific antigens, isolated and purified as mentioned in Examples 10 and 14 were prepared essentially as described in Example 3, beginning from the step of vacuum drying.
Monoclonal antibodies

Balb/c mice were immunized three times intraperitoneally with 100 g S. chartarum specific antigen at two weeks intervals. Freud's complete adjuvant was used for the first injection and Freud's incomplete adjuvant for the second injection. The last injection was given without adjuvant, and the mice were sacrificed four days later. monoclonal antibodies were produced according to Galfre, G., et al., Nature 266:550-552 (1977)). Spleen cells of immunized mice were fused with the NSI-1 mouse myeloma cell line using 50 % polyethylene glycol (MW 1500). Hybridomas were tested for antibody production with ELISA and immunoblotting methods and selected hybridomas were cloned twice by the limiting dilution method.

Example 16
Preparing dip-stick kits

A narrow zone of a nitrocellulose strip (strip 1, approximately 6 x 25 mm) is coated with a monoclonal antibody against specific components of Stachybotrys chartarum. Coloured latex particles are coated with another antibody against the same component. The coated latex particles are dried on a zone in the middle of a strip of absorbing polyethylene material (STRIP 2). The diameter of the latex particles is small enough that they could flow freely through the pores in both strip materials. The two strips are attached on a plastic backing so that they are in a contact which allows capillary flow of sample liquid from strip 2 to strip 1 when the end of strip 2 is dipped into liquid. For absorption of excess liquid, a pad of filter paper is attached in contact with strip 1 opposite to strip 2. The dipstick constructed is used to perform the rapid indicator fungus component test according to the following instructions.

Example 17
Collection of environmental samples

The collection and preparation of environmental samples is very simple. An investigator evaluates and recognizes suitable sites of sampling that represent contaminated area sufficiently well. a subsample is taken from a sampling site (building material, other substrate, accumulated dust, etc) and is transferred into a test tube containing buffer solution and shaken carefully. A dip-stick is placed into the buffer and the instruction in Example 19 is followed.

Example 18
Setting up a test protocol

During the development of the test, samples from pure cultures of various Stachybotrys
chartarum strains and other indicator fungal strains and other common non-indicator fungal species as well as mixed cultures of the indicator and other fungi and contaminated environmental samples are prepared artificially with known fungal species and collected from field conditions.

Example 19
Performance of the immunochromatographic test
1. The samples obtained as described in Example 17 are diluted in buffer (PBS containing Tween 20 and aprotinin (50 kIU/ml), pH 7.4)

2. One end of the dipstick (end of strip 2) is dipped into the diluted sample and kept there until the liquid front reaches strip 1, and is then removed from the sample.

3. During an incubation of 5 minutes the sample is allowed to migrate in the strips and the latex particles are transferred with the liquid over the antibody-coated zone to the other end of the dipstick.

4. Strip 1 is inspected. Already a minimal amount of S. chartarum components will cause a visible line. If a coloured zone is formed, the result is interpreted as positive.

Data obtained by monoclonal antibody based S. chartarum-dipstick test can be compared with a corresponding ELISA analysis. Increased ELISA levels will reflect positive dipstick analysis. Positive dip-stick results are associated with health hazardous contamination of S. chartarum and indicate a possibility of contamination with other harmful molds.
Table 1. Crossreactivity of *Stachybotrys chartarum* components (cultured in cellulose broth)

<table>
<thead>
<tr>
<th>Inhibitory concentration 200 µg/ml</th>
<th>110</th>
<th>85</th>
<th>82</th>
<th>70</th>
<th>66</th>
<th>62</th>
<th>60</th>
<th>55</th>
<th>50</th>
<th>42</th>
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<th>27</th>
<th>20</th>
<th>14</th>
<th>kDa</th>
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<td>+</td>
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<tr>
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<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
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<td>+</td>
<td>-</td>
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+++ = strong crossreactivity, ++ = moderate crossreactivity, + = slight crossreactivity, - = no crossreactivity.
We claim:

1. An antigenic component, characterized in that it is capable of eliciting antibodies, which are specific for one or more defined indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi, said antigenic component being obtainable by the steps of:

   (a) isolating antigenic components from one or more indicator fungi

   (b) determining the cross-reactivity of the antigenic components of step a) with antigenic components from other non-indicator fungi;

   (c) selecting those antigenic components which are specific for the indicator fungi and substantially devoid of cross-reactivity with other non-indicator fungi.

2. The antigenic component according to claim 1, wherein the indicator fungi are selected from the genera *Stachybotrys*, *Aspergillus* and *Memnoniella*.

3. The antigenic component according to claims 1-2, wherein the indicator fungi is *Stachybotrys chartarum*.

4. The antigenic component according to claims 1-2, wherein the indicator fungi is *Aspergillus versicolor*.

5. The antigenic component according to claims 1-2, wherein the indicator fungi is *Memnoniella echinata*.

6. The antigenic components according to claims 1-5, wherein the antigenic components are produced by recombinant DNA techniques.

7. The use antigenic components according to claims 1-6 for manufacturing test kits for detecting the presence or absence of the selected indicator fungi or components thereof.

8. The use antigenic components according to claims 1-6 for manufacturing test kits for evaluating the quality of fungal contamination, remedial measures needed and checking the success of applied remedial measures.
9. The use antigenic components according to claims 1-6 for manufacturing test kits for screening the extent of fungal contamination, remedial measures needed and checking the success of applied remedial measures.

10. The use of antigenic components of claims 1-6 for manufacturing test kits for assessing whether a person has been exposed to contamination from an indicator fungus and/or whether the cause of an allergic reaction is exposure to an the indicator fungus by determining the presence or absence of antibodies in serum samples from the person suspected of having been exposed to contamination.

11. The use of antigenic components according to claims 1-6 for producing binding substances which specifically recognize and bind antigenic components from indicator fungi and which do not recognize and bind to antigenic components of other fungi.

12. The use of binding substances specifically recognizing and recognizing and binding to at least one antigenic component according to claim 1-6, which is specific for one or more indicator fungi and which is substantially devoid of cross-reactivity with other fungi for manufacturing test kits for evaluating the quality and extent of fungal contamination, remedial measures needed and checking the success of applied remedial measures.

13. The use according to any of claims 11-12, wherein the binding substances are selected from binding peptides, polyclonal antibodies, monoclonal antibodies and/or fragments of said polyclonal or monoclonal antibodies.

14. A method for detecting the presence or absence of selected indicator fungi, wherein the detection is performed as an immunochemical assay comprising the steps of:

(a) obtaining an environmental sample and preparing a subsample suspension; by diluting and/or extracting the solid environmental samples;

(b) contacting said subsample with one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific for at least component of an indicator fungus and which is substantially devoid of cross-reactivity with other non-indicator fungi;
15. A method for evaluating the quality of fungal contamination, remedial measures needed and checking the success of applied remedial measures, wherein the detection is performed as an immunochemical assay comprising the steps of:

(a) obtaining a solid or liquid environmental sample and preparing a subsample suspension by optional diluting or extracting;

(b) contacting said subsample with one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific for at component of an indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi; and

(c) recording the presence or absence of the indicator fungi in the environmental sample either by direct or indirect means.

16. A method for screening the extent of fungal contamination, remedial measures needed and controlling the success of applied remedial measures, wherein the detection is performed as an immunochemical assay comprising the steps of:

a) obtaining two or more environmental samples from different areas suspected of contamination;

b) contacting said samples with one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific for at component of an indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi; and

c) recording the presence or absence of the indicator fungi in the environmental sample either by direct or indirect means.

17. The method according to claims 15 or 16 characterized in that for checking the success of applied remedial measures the sampling is repeated, at least once, often with suitable intervals.

18. A test kit for detecting the presence or absence of selected indicator fungi with the method of claim 14, wherein the test kit comprises one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific
for at least one indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi.

19. A test kit for evaluating the quality fungal contamination, remedial measures needed and controlling the success of applied remedial measures with the methods of claim 15, wherein the test kit comprises one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific for at least one indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi.

20. A test kit for screening the extent fungal contamination, remedial measures needed and controlling the success of applied remedial measures with the methods of claim 10, wherein the test kit comprises one or more binding substances capable of specifically recognizing at least one antigenic component, which is specific for at least one indicator fungi and which is substantially devoid of cross-reactivity with other non-indicator fungi.
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/569, C12Q 1/00, C07K 16/14, C07K 14/37
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
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  "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search: 8 December 2000

Date of mailing of the international search report: 3-12-2000

Name and mailing address of the ISA/Swedish Patent Office
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Facsimile No. +46 8 666 02 86

Authorized officer:
Carl-Olof Gustafsson/GH
Telephone No. +46 8 782 25 00

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