# IMMUNOCHEMICAL DETECTION OF MUCORALES SPECIES IN FOODS

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## SUMMARY

In this paper, immunological detection of fungi of the order Mucorales in food is reviewed. The Enzyme Linked Immunosorbent Assay (ELISA), based on the immunochemical properties of the extracellular polysaccharides (EPS), has been found to be specific for species belonging to Mucorales. Testing of 161 food samples demonstrated that this method has several advantages compared with other detection methods, including colony count. The ELISA method was found to be sensitive, rapid and reliable for a number of food products. Checks for false positive reactions are necessary for samples of walnuts and jams. In general, the presence in food at some time of species belonging to Mucorales can be established using this ELISA assay, even if the mould itself is inactivated or removed by filtration.

## INTRODUCTION

Mycological detection of species belonging to the order Mucorales in food is important because of their wide distribution in stored grain, fruits and vegetables, which leads in many cases to food spoilage (Pitt and Hocking, 1985). Medically, species belonging to Mucorales are important for their ability to cause mucormycosis in man (Kaufman *et al.*, 1989; De Ruiter *et al.*, 1991a). Testing of processed foods is often necessary to verify if the food was prepared from high quality raw materials. Conventional detection methods have been summarised by Jarvis *et al.* (1983). The mould colony count is used in many countries as a standard method prescribed in food legislation. However, this method cannot be used for processed foods in which the mould itself has been killed or removed. In addition, the mould colony count method lacks precision, is time consuming and is not specific for species belonging to Mucorales.

To overcome the disadvantages of conventional methods, immunological detection methods based on the immunochemically active extracellular polysaccharides (EPS) have been developed for various genera of fungi.

*Aspergillus* and *Penicillium* species could be detected either by an enzyme-linked immunosorbent assay (ELISA) or a latex agglutination assay (Notermans and Soentoro, 1986; Kamphuis *et al.*, 1989). It was found that EPS antigens involved have ß(1,5)-linked galactofuranose oligomeric epitopes, and that they are heat stable and water extractable, enabling detection of these moulds in food even after heat treatment or thermal processing (Kamphuis and Notermans, 1992).

A successful immunoassay for species of Mucorales requires that the antigens are easily extractable from food and that they be stable towards heating and other processes which inactivate fungi. Furthermore, the excreted immunologically active EPS must also be genus, or at least order, specific. For quantitative purposes the amount of excreted EPS and the amount of fungal biomass as well as the ELISA titre should be related. Also, it must be possible to recognise any false positive reaction which may occur as a result of the complexity of the food samples.

This paper reviews the features important for the immunological detection in food of fungi in Mucorales, including the genera *Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Syncephalastrum* and *Thamnidium*. An ELISA assay developed for detection of these fungi was tested using a variety of food samples.

### The extracellular polysaccharides of Mucorales

The immunochemical properties of polysaccharides excreted by species of Mucorales have been established partly by Miyazaki and Irino (1972) and Miyazaki *et al.* (1980). EPS are mainly composed of glucuronic acid, mannose, fucose, glucose, galactose and minor amounts of protein, and they are water extractable and resistant towards heating (Hough and Perry, 1955; Martin and Adams, 1956; De Ruiter *et al.*, 1991a, 1992). Immunological detection methods based on the immunochemically active EPS have been developed for detection of these moulds (Jones and Kaufman, 1978; Notermans and Heuvelman, 1985; Lin and Cousin, 1987; Kaufman *et al.*, 1989). Specific blocking of the antibody, which enables detection of false positive reactions, requires structural information about the epitopes. Although no detailed knowledge about the epitopes of *Mucor* EPS is available, inhibition using high amounts of methyl-α-mannosides can be performed as described by Notermans *et al.* (1988).

## METHODS

### Determination of EPS and biomass production

Moulds used in this study were grown at their optimal temperature in shaken submerged cultures for 7 days in a basal medium of yeast nitrogen base (YNB, Difco Laboratories, Detroit, USA) supplemented with 30 g/L glucose as carbon source. Extracellular polysaccharides (EPS) of the moulds were isolated from the culture fluid after filtration of the mycelium and purified by ethanol precipitation as described by De Ruiter *et al.* (1991b). The yield of biomass was measured after drying the extracted mycelium in an oven at 80°C overnight.

### The *Mucor* sandwich ELISA

A sandwich ELISA assay with polyclonal IgG antibodies raised in rabbits against extracellular polysaccharides from *Mucor racemosus* was performed as described by Notermans and Heuvelman (1985) and De Ruiter *et al.* (1991b). The ELISA reactivity is expressed as the titre, which is the reciprocal dilution of the sample just giving a positive reaction, i.e. an extinction $\geq 0.1$.

### Specificity of the ELISA for species belonging to Mucorales

Notermans and Heuvelman (1985) and Notermans *et al.* (1986) established a complete cross reactivity in a sandwich ELISA between species of the genera *Mucor* and *Rhizopus* with antibodies raised against EPS of *Mucor racemosus*. No cross reactivity occurred with the major species of *Penicillium* and *Aspergillus* (Notermans and Soentoro, 1986). Antigenic similarity between some species of *Rhizopus* was also established by Polonelli *et al.* (1988). Species of other genera belonging to Mucorales were tested for their ability to react with antibodies raised against EPS of *Mucor racemosus*.



### The use of the *Mucor* ELISA in food samples

In order to study the effectiveness of the *Mucor hiemalis* ELISA assay for detecting species belonging to Mucorales, 161 food samples were tested. To 10 g of sample 90 mL of a 0.07 M phosphate buffered saline (0.15 M) solution, pH 7.2, was added. The mixture was homogenised in a Colworth 400 Stomacher for 1 min, followed by centrifugation (15 min at 3000 rpm). The supernatant was used for all experiments.

The results of the ELISA were roughly compared with colony counts performed by the Netherlands Food Inspection Laboratories of Rotterdam, Zutphen and Utrecht. Fungal colonies were enumerated on Dichloran 18% Glycerol agar (DG18) as described by Hocking and Pitt (1980).

## RESULTS

### Specificity of the ELISA for species belonging to Mucorales

As shown in Table 1, EPS from 10 species belonging to six genera of Mucorales reacted to the ELISA system using EPS of *Mucor racemosus*. It appears that some EPS fractions are more reactive than others.

**Table 1. ELISA reactivity of isolated EPS of various species of Mucorales**

| Species | Strain | EPS yield (mg/l)[1] | ELISA titre[2] |
|---|---|---|---|
| *Mucor racemosus* | RIVM H473-R5 | 190 | 400 |
| *Mucor hiemalis* | CBS 201.28 | 378 | 220 |
| *Mucor circinelloides* | RIVM 40 | 498 | 560 |
| *Rhizopus oryzae* | LUW 581 | 626 | 50 |
| *Rhizopus stolonifer* | CBS 609.82 | 300 | 140 |
| *Rhizomucor miehei* | CBS 371.71 | 324 | 610 |
| *Rhizomucor pusillus* | CBS 432.78 | 220 | 730 |
| *Absidia corymbifera* | LUW 017 | 449 | 50 |
| *Syncephalastrum racemosum* | CBS 443.59 | 504 | 240 |
| *Thamnidium elegans* | CBS 342.55 | 46 | 70 |

[1] The yield of EPS is expressed as the isolated amount in milligrams per litre of culture medium.
[2] The ELISA titre is expressed as the reciprocal dilution of a 10 μg/ml solution of EPS in distilled water, just giving a positive reaction, compared to a blank, in the ELISA as described in Notermans *et al.* (1986). It can be used to determine the immunochemical reactivity of the isolated EPS fractions.

### Relation between EPS production, biomass and ELISA titre

It was demonstrated that the excreted amount of EPS can be considered as a marker for the amount of biomass of *Mucor hiemalis*. For *M. hiemalis* grown on YNB medium with glucose, the ratio between the amount of excreted EPS, both determined by isolation and ELISA, and the fungal biomass varied between 0.1 and 0.4 (mg/mg) depending on carbon source and growth phase. This was valid also for the EPS of the 10 species listed in Table 1 (De Ruiter *et al.*, 1991a). In addition to the immunochemically active EPS it was found that other neutral polysaccharides could be excreted depending on species and carbon source used for growth (De Ruiter *et al.*, 1991b).

Table 2. *Mucor* ELISA titres on food samples with fungal counts of less than 200 cfu/g

| Food | Samples tested | *Mucor* ELISA titre[1] | | | |
|---|---|---|---|---|---|
| | | -[2] | + | ++ | >+++ |
| **Juices** | | | | | |
| Grape juice | 6 | - | - | 5 | 1 |
| Apple juice | 7 | 7 | - | - | - |
| Pineapple juice | 5 | 5 | - | - | - |
| Blackcurrant juice | 6 | 1 | 1 | 3 | 1 |
| Orange juice | 5 | 5 | - | - | - |
| Mixed juices | 7 | 6 | - | 1 | - |
| Tomato juice | 3 | 3 | - | - | - |
| **Spices** | | | | | |
| Nutmeg | 3 | - | - | 3 | - |
| Paprika powder | 2 | - | - | - | 2 |
| Mixed spices | 2 | 1 | 1 | - | - |
| **Nuts** | | | | | |
| Pistachio | 2 | 2 | - | - | - |
| Pistachio[3] | 2 | 1 | - | 1 | - |
| Hazelnuts | 6 | 6 | - | - | - |
| Roasted peanuts | 9 | 9 | - | - | - |
| Peanut butter | 11 | 9 | - | - | 2 |
| Raw peanuts | 3 | 3 | - | - | - |
| **Flour** | | | | | |
| Maize flour | 3 | 3 | - | - | - |
| Buckwheat | 1 | 1 | - | - | - |
| Wheat flour | 3 | 3 | - | - | - |
| **Dried fruits** | | | | | |
| Dates | 9 | 9 | - | - | - |
| Figs | 7 | 7 | - | - | - |
| Apricots | 4 | 4 | - | - | - |
| Raisins | 1 | 1 | - | - | - |

[1] *Mucor* ELISA titre is expressed as reciprocal dilution just giving a positive ELISA reaction, compared to the blank as described in Notermans *et al.* (1986).
[2] -, lower than 20; +, between 20 and 100; ++, between 100 and 1000; >+++, higher than 1000
[3] Results taken from Notermans *et al.* (1988).



### The use of *Mucor* ELISA in food samples

The comparisons between ELISA results, expressed as the ELISA titre, and counts on DG18 are given in Tables 2 and 3. Samples with low counts, i.e. less than 200 cfu/g, are summarised in Table 2, while Table 3 summarises those with higher counts. Only samples with no false positive ELISA reactions are included in the tables: jams, which frequently showed false positive reactions, have been excluded.

As shown in Table 2, some pasteurised juices, mainly grape juices and blackcurrant juices, gave high *Mucor* ELISA titres. It is to be expected that the fruits used as raw materials for these juices would be contaminated with moulds belonging to Mucorales. Juices from fruits in which no contamination with these moulds is to be expected, e.g. orange, pineapple and tomato, did not give a positive ELISA reaction.

Spices, in which enumeration detected no fungal contamination, gave rise to high ELISA titres, indicating the previous presence of moulds. Most spices entering Europe are fumigated. Nuts, flours and dried fruits show low *Mucor* ELISA titres.

Table 3 shows results for samples which gave high colony counts on DG18. Counts are for total fungi, not specifically Mucorales: the number of samples in which Mucorales were detected on DG18 is given in the last column.

Table 3. *Mucor* ELISA titres on various food samples with relatively high mould counts

| Food | Samples tested | *Mucor* ELISA titre[1] | | | | $Log_{10}$ cfu/g | | | Samples containing Mucorales[4] |
|---|---|---|---|---|---|---|---|---|---|
| | | -[2] | + | ++ | >+++ | 2-<3 | 3-<4 | >4 | |
| **Spices** | | | | | | | | | |
| Nutmeg | 8 | - | - | 2 | 6 | 2 | 4 | 2 | - |
| Nutmeg[3] | 1 | - | - | - | 1 | - | - | 1 | - |
| Paprika powder | 5 | - | - | - | 5 | - | 3 | 2 | 1 |
| Mixed spices | 2 | 2 | - | - | - | - | - | 2 | - |
| Mixed spices[3] | 4 | - | - | - | 4 | - | - | 4 | 2 |
| **Nuts** | | | | | | | | | |
| Pistachios | 2 | 1 | 1 | - | - | - | - | 2 | - |
| Pistachios[3] | 1 | - | - | 1 | - | 1 | - | - | - |
| Hazelnuts | 12 | 10 | 1 | - | 1 | 9 | 3 | - | 4 |
| Hazelnuts[3] | 3 | 1 | 2 | - | - | - | 3 | - | - |
| Roasted peanuts | 1 | 1 | - | - | - | 1 | - | - | - |
| Raw peanuts | 2 | 2 | - | - | - | 1 | 1 | - | 1 |
| **Flour** | | | | | | | | | |
| Maize flour | 5 | 1 | - | 3 | 1 | 1 | 4 | - | 1 |
| Oatmeal | 2 | - | 2 | - | - | 1 | 1 | - | - |
| Barley meal | 2 | 1 | - | - | 1 | - | 2 | - | 1 |
| Buckwheat | 1 | - | - | - | 1 | - | - | 1 | 1 |
| **Dried fruits** | | | | | | | | | |
| Figs | 1 | 1 | - | - | - | 1 | - | - | - |
| Currants | 2 | 1 | - | - | 1 | 1 | 1 | - | - |

[1, 2, 3] see Table 2.
[4] Number of samples in which species belonging to Mucorales were detected on DG18.

Most spices in Table 3 were fairly heavily contaminated with fungi, and had very high *Mucor* ELISA titres. However, in only 3 of these 20 samples were species of Mucorales found on DG18. Eighteen samples of walnuts had high false positive ELISA titres and are not included. Notermans *et al.* (1988) and Kamphuis *et al.* (1989) reported similar problems with walnuts in studies on immunological detection of *Penicillium* and *Aspergillus* species. On the other hand samples of pistachios, hazelnuts and peanuts showed low ELISA titres in the presence of relatively high total mould counts, including 5 samples in which species of Mucorales could be detected.

Samples of flour showed a good correlation between colony count and ELISA titre for species of Mucorales. In the three flour samples with high (>+++) *Mucor* ELISA titres, species of Mucorales were detected on DG18.

## DISCUSSION

In this study, the possibility of using an ELISA technique to detect mould species belonging to Mucorales in food was tested on 161 food samples. The *Mucor heimalis* ELISA was shown to react with 11 species in six genera of Mucorales. Colony counts did not always correlate well with the ELISA test, but DG18 is not well suited to the isolation of Mucorales.

This ELISA method appears to have several advantages compared with other mould detection methods, including the colony count. It was found to be sensitive, rapid and reliable for a number of food products. The earlier occurrence of species belonging to Mucorales can be established using this ELISA assay, even if the mould itself is inactivated or removed by filtration. This was shown in pasteurised juices, in which the *Mucor* ELISA can be used to determine the quality of fruit used as raw materials for the production of these juices. Therefore the ELISA provides information on the history of mould contamination of raw materials, which is important in the food industry.

In flour samples there was a good correlation between the colony counts and the *Mucor* ELISA titre. The use of this ELISA test for samples of walnuts and jams should be further examined, because of the high number of false positive reactions that occurred.

The reliability of the *Mucor* ELISA could be improved by elucidation of the structure of epitopes involved in the immunochemical reaction. Research is in progress to gain more detailed structural information about the immunogenic properties of EPS produced by species of Mucorales.

It can be speculated that the specificity can be extended to a large number of species belonging to Mucorales. It has been assumed in this study that EPS derived from species of Mucorales have common antigenic determinants, which are specific and characteristic for this order.

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