J. Microbiol. Biotechnol. (2004), 14(2), 385–389

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Detection of *Aspergillus*, *Penicillium*, and *Fusarium* Species by Sandwich Enzyme-Linked Immunosorbent Assay Using Mixed Monoclonal Antibodies

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Received: December 11, 2002
Accepted: October 2, 2003

Abstract The antibody-mix sandwich enzyme-linked immunosorbent assay (Ab-mix sELISA) system was developed in order to simultaneously detect the extracellular polysaccharide (EPS) of *Aspergillus*, *Penicillium*, or *Fusarium* species using one detection system. The detection limit and detection range of the Ab-mix sELISA towards EPS of *Penicilliun citrinum* were not changed, and those towards *Fusarium moniliforme* EPS were changed a little compared to that of individual sandwich ELISA [9, 10]. The fungal culture filtrates of *Aspergillus* and *Penicillium* species showed nearly similar reactivity towards Ab-mix sELISA as that of sELISA using the MAb 1G11 alone [9]. Also, the fungal culture filtrates of *Fusarium* species showed nearly the same reactivity towards Ab-mix sELISA as that of sELISA using the MAb 1B8 alone [10]. Thus, this ELISA system showed that the three genera of molds, *Aspergillus*, *Penicillium*, or *Fusarium*, which are three major important molds producing mycotoxins in food or agricultural commodities, could be detected at the same time, using one detection system.

Key words: Antibody-mix, sandwich ELISA, *Aspergillus*, *Penicillium*, *Fusarium*, extracellular polysacharide, monoclonal antibody

Molds are widely distributed in the environment [17]. Mold contamination not only causes deterioration of foods and feeds, but can also adversely affect the health of humans and animals, since they may produce toxic metabolites called mycotoxins [1, 16]. The three major important genera of mycotoxin-producing fungi in foods are *Aspergillus*, *Penicillium*, and *Fusarium* [2], and it is very important in detecting the molds in agricultural commodities, food. or feed. Many different types of mold assays have been used for detecting these molds [2, 3, 5–7, 19], however, most of them are inaccurate or time- and labor-consuming and/or not reproducible as well [2, 7]. For example, direct and dilution plating method with subsequent identification by a microscope takes up to 7 days or more, and is inaccurate and detects only viable organisms. Suitable assays for molds, therefore, are required to implement control and regulatory strategies and to develop appropriate feeding regiments for mold-infested feeds.

Since Notermans and Heuvelman [14] have shown the possibilities for enzyme linked-immunosorbent assay (ELISA) in simply and accurately detecting mold in food or agricultural commodities, many attempts have been made to develop ELISA systems for detecting the mold contaminated in food and agricultural commodities [13, 15, 21]. We produced a polyclonal antibody which was specific to the extracellular polysaccharide (EPS) of *Aspergillus* and *Penicillium* species and developed sandwich ELISA [8]. Also, monoclonal antibody (MAb) 1G11, which was specific to the EPS of *Aspergillus* and *Penicillium* species [9], and MAb 1B8, which was specific to the EPS of *Fusarium* species, were produced and individual sandwich ELISAs were developed [10].

De Ruiter *et al.* [4] pointed out that the detection of molds in food or agricultural commodities by the ELISA had the disadvantage of detecting only one genus or related species, because the antibody was specific only to the one genus or related species. Therefore, detecting several species of molds at the same time by ELISA is needed. Wyatt *et al.* [20] demonstrated the possibility of sandwich ELISA for simultaneously detecting various *Salmonella* species by using polyclonal Ab for the capture stage and a cocktail of MAbs for the detector stage. The assay recognized a wide range of *Salmonella* serotypes. Yong and Cousin [21] produced polyclonal antibody from rabbits immunized with a mixture of several fungal mycelia, and each fungal mycelum showed reactivity towards the polyclonal antibody. Robertson and Patel [18] also produced polyclonal antibody from rabbit immunized with a mixture of 5 kinds

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of molds, and Nielsen *et al.* [12] and Mousing *et al.* [11] employed a mixture of *Salmonella* lipopolysaccharide as coating antigens for screening large number of muscle fluid and serum sample of pigs in an indirect ELISA (the mix-ELISA). Nevertheless, there has not yet been any reported attempt to detect several genera of molds at the same time, employing a mixture of MAbs for the capture stage and a cocktail of MAbs for the detector stage (Ab-mix sELISA).

This paper reports the possibility of an Ab-mix sELISA system for detecting the three genera of molds (*Aspergillus*, *Penicillium*, and *Fusarium*) with one ELISA system, using the MAbs 1G11 and 1B8.

Maintenance of mold and preparation of mold antigens are described elsewhere [8]. The purification of MAb 1G11 and conjugation of MAb 1G11 and horseradish peroxidase (HRP) [9], and the purification of MAb 1B8 and conjugation of MAb 1B8 and HRP were described elsewhere [10]. The fungal culture filtrate was produced according to the method described in the previous report [8]. An Ab-mix sELISA was performed as follows; Purified MAb 1G11 (2 μg/ml) and MAb 1G8 (2 μg/ml) were thoroughly mixed, and 100 μl of the MAbs mixture in coating buffer (0.05 M Tris(hydroxymethyl)amino methane buffer, pH 9.0) were dispensed into the wells of 96-well microplate (Maxisorp^T, Nunc Co., Roskilde, Denmark) and kept overnight at 4°C for coating. After washing each well three times with 150 μl of phosphate buffered saline with Tween 20 (PBST: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20), and tapping the plate onto paper towel to remove the remaining liquid, the 100 μl of *F. moniliforme* EPS or *P. citrinum* (up to 10 μg/ml) as a standard or broth of liquid culture of each fungi diluted up to 100,000 times were added into the wells, followed by reaction for 1 h at room temperature. Then, the wells were washed in the same way as the above and 100 μl of a mixture of MAb 1G11-HRP and MAb 1B8-HRP conjugates, both of which were diluted 4,000 times, was added into the wells and reacted for 1 h at room temperature. After washing the wells again by the above method, 100 μl of fresh substrate solution (0.01% 3,3'5,5'-tetramethyl benzidine, 0.05 M phosphate citrate buffer, pH 5.0, and 1% H_2O_2 added to the final 0.001%) were added into each well and reacted for 30 min at room temperature. The enzyme reaction was stopped by adding 50 μl of the stop solution (2 M H_2SO_4). A reading was carried out at 450 nm with a microplate reader (THERMOmax™ Molecular Devices Co., Sunnyvale, CA, U.S.A.) and the average value was obtained from the three wells per each treatment.

A standard curve of Ab-mix sELISA for EPS of *P. citrinum* or EPS of *F. moniliforme* is shown in Fig. 1. The detection limit and detection range of the Ab-mix sELISA towards the *F. moniliforme* EPS were not changed, compared to those of the standard curve of sandwich

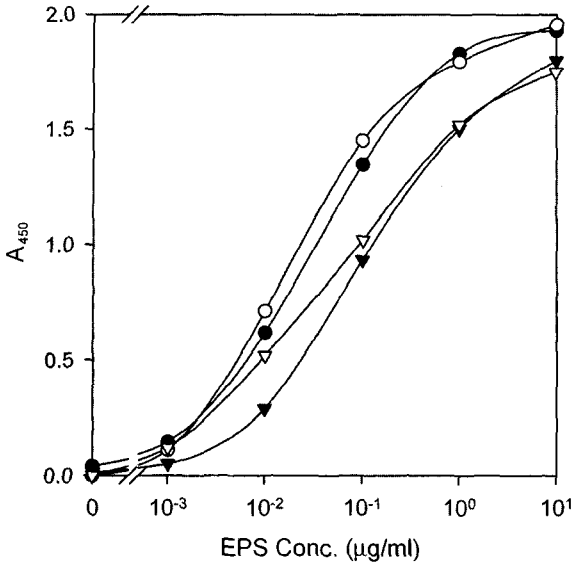

Fig. 1. A standard curve of antibody-mix sandwich ELISA for detecting the extracellular polysaccharide (EPS) using monoclonal antibodies 1G11 and 1B8.
-●- EPS of *Fusarium moniliforme* in antibody-mix sandwich ELISA, -○- EPS of *Fusarium moniliforme* in sandwich ELISA using MAb 1B8 in the previous report [10], -▼- EPS of *Penicillium citrinum* in antibody-mix sandwich ELISA, -▽- EPS of *Penicillium citrinum* in sandwich ELISA using MAb 1G11 in the previous report [9]. An antibody-mix sandwich ELISA was done as follows: MAb 1G11 reactive to the EPS of *Aspergillus* and *Penicillium* species and MAb 1B8 reactive to the EPS of *Fusarium* species were mixed well and coated on the microplate. After washing, the EPS of *Fusarium moniliforme* or *Penicillium citrinum* was added into the well and then reacted for 1 h at room temperature. Then, a mixture of MAb 1G11-horseradish peroxidase and MAb 1B8-horsseradish peroxidase was added and reacted for 1 h at room temperature. The coloring reaction was done and the absorbance at 450 nm was measured with a microplate reader.

ELISA using MAb 1B8 [10], and the detection limit of *P. citriinum* EPS towards Ab-mix sELISA was little changed (1 ng/ml→3 ng/ml). This demonstrates that the EPS of *P. citrinum* and *F. moniliforme* could be simultaneously detected by the Ab-mix sELISA using one detection system. In the previous study [9], the *A. flavus* EPS was used as the standard in the individual sandwich ELISA using MAb 1G11 alone to detect the EPS of *Aspergillus* and *Penicillium* species. In Ab-mix sELISA, *P. citrinum* EPS was used as a standard, because *A. flavus* EPS showed reactivity towards the sandwich ELISA with MAb 1B8 alone and also the sandwich ELISA with MAb 1G11 alone [9]. In the previous study [10], *P. citrinum* EPS showed no reactivity towards sandwich ELISA with MAb 1B8 alone. On the other hand, *F. moniliforme* EPS was used as a standard for Ab-mix sELISA, because it showed reactivity towards only MAb 1B8 in the individual sandwich ELISA [10].

Reactivities of mold culture broth, which was diluted serially with PBST, towards the antibody-mixture of sandwich ELISA using MAb 1G11 and 1B8 were determined by the Ab-mix sELISA (Table 1). The fungal culture filtrates of

Aspergillus and *Penicillium* species showed almost the same reactivities towards Ab-mix sELISA as those of sandwich ELISA using MAb 1G11 alone. Although the culture filtrates of *A. phoenicis*, *A. usamii* mut. *shiro-usamii*, *P. expansum*, *P. glabrum*, and *P. spinulosum* did not show reactivities when they were diluted 100,000 times, they showed reactivities, when diluted less than 10,000 times. Also, the fungal culture filtrates of *Fusarium* species showed almost the same reactivity towards Ab-mix sELISA as those of sandwich ELISA using MAb 1B8 alone. Although the culture filtrate of only *F. graminearum* did not show reactivity when diluted 100,000 times, it showed reactivity when diluted 10,000 times, and the other culture filtrates of *Fusarium* species showed similar reactivities. The culture filtrates of *Cladosporium resinae* and *Trichoderma viride* also showed similar reactivities towards Ab-mix sELISA. Even though some of the filtrates of the three genera of fungi showed slightly lower reactivities than those of individual sandwich ELISA, they could still be detected when they were diluted less than 10,000 times. This signifies that the Ab-mix sELISA can detect the EPS of the test fungal culture without losing the reactivities, compared to those of the individual sandwich ELISA using MAb 1G11 or MAb 1B8 alone, suggesting that the Ab-mix sELISA with the two MAbs developed in this study could be used for simply and rapidly detecting the three genera of mold, *Aspergillus*, *Penicillium*, and *Fusarium*, simultaneously using one ELISA system.

Yong and Cousin [21] developed a nonspecific sandwich ELISA to detect a broad-spectrum of molds in

Table 1. Comparison of the reactivity towards fungal culture broths as determined by the antibody-mix sandwich ELISA[1] and individual sandwich ELISA in the previous paper [9, 10].

Microorganism	Dilution rate[3]											
	100			1 K			10 K			100 K		
1 *Aspergillus awamori* (6915)[2]	+[4]	+[5]	+[6]	+[4]	+[5]	+[6]	+[4]	+[5]	-[6]	-[4]	-[5]	-[6]
2 *Aspergillus awamori* var. *femeus* (6902)	+	+	-	+	+	-	+	+	-	-	-	-
3 *Aspergillus candidus* (6006)	-	-	-	-	-	-	-	-	-	-	-	-
4 *Aspergillus carbonarius* (6913)	+	+	+	+	+	-	+	+	-	-	-	-
5 *Aspergillus clavatus* (6033)	+	+	+	+	+	-	+	+	-	-	-	-
6 *Aspergillus ficuum* (6134)	+	+	-	+	+	-	+	+	-	-	-	-
7 *Aspergillus flavus* (6961)	+	+	+	+	+	+	+	+	-	+	+	-
8 *Aspergillus foetidus* (6906)	+	+	-	+	+	-	+	+	-	-	-	-
9 *Aspergillus fumigatus* (6145)	+	+	-	+	+	-	+	+	-	-	-	-
10 *Aspergillus nidulans* (6981)	-	-	-	-	-	-	-	-	-	-	-	-
11 *Aspergillus nidulans* var. *roseus* (6058)	+	+	+	+	+	+	+	+	-	+	+	-
12 *Aspergillus niger* (6910)	+	+	-	+	+	-	-	-	-	-	-	-
13 *Aspergillus niger* var. *macrosporus* (6035)	+	+	+	+	+	-	+	+	-	-	-	-
14 *Aspergillus oryzae* var. *oryzae* (6983)	+	+	+	+	+	+	+	+	-	-	-	-
15 *Aspergillus parasiticus* (6170)	+	+	+	+	+	+	+	+	-	-	-	-
16 *Aspergillus phoenicis* (6908)	+	+	-	+	+	-	+	+	-	-	+	-
17 *Aspergillus usamii* mut. *shiro-usamii* (6954)	+	+	-	+	+	-	+	+	-	-	+	-
18 *Aspergillus versicolor* (6987)	+	+	+	+	+	+	+	+	+	+	+	+
19 *Penicillium aurantiogriseum* var. *viridicatum* (6117)	+	+	-	+	+	-	+	+	-	-	-	-
20 *Penicillium camembertii* (6102)	+	+	+	+	+	+	+	+	-	-	-	-
21 *Penicillium caseicolum* (6041)	+	+	-	+	+	-	+	+	-	-	-	-
22 *Penicillium chrysogenum* (6053)	+	+	-	+	+	-	+	+	-	+	+	-
23 *Penicillium citrinum* (6927)	+	+	-	+	+	-	+	+	-	+	+	-
24 *Penicillium claviforme* (6267)	+	+	-	+	+	-	+	+	-	+	+	-
25 *Penicillium decumbens* (6109)	+	+	-	+	+	-	+	+	-	+	+	-
26 *Penicillium echinulatum* (6402)	+	+	-	+	+	-	+	+	-	-	-	-
27 *Penicillium expansum* (6434)	+	+	-	+	+	-	+	+	-	-	+	-
28 *Penicillium glabrum* (6930)	+	+	-	+	+	-	+	+	-	-	+	-
29 *Penicillium islandicum* (6405)	-	-	-	-	-	-	-	-	-	-	-	-
30 *Penicillium oxalicum* (6113)	+	+	-	+	+	-	+	+	-	-	-	-
31 *Penicillium pinophilum* (7001)	+	+	-	-	-	-	-	-	-	-	-	-
32 *Penicillium purpurogenum* (6118)	+	+	-	+	+	-	+	+	-	-	-	-
33 *Penicillium roquefortii* (6080)	+	+	-	+	+	-	+	+	-	+	+	-
34 *Penicillium spinulosum* (6442)	+	+	-	+	+	-	+	+	-	-	+	-

Table 1. Continued.

Microorganism	Dilution rate[3]											
	100			1 K			10 K			100 K		
35 *Fusarium flocciferum* (6107)[2]	+[4]	-[5]	+[6]	+[4]	-[5]	+[6]	+[4]	-[5]	+[6]	+[4]	-[5]	+[6]
36 *Fusarium graminearum* (6150)	+	-	+	+	-	+	+	-	+	-	-	+
37 *Fusarium merismoides* (6153)	+	-	+	+	-	+	+	-	+	+	-	+
38 *Fusarium moniliforme* (6149)	+	-	+	+	-	+	+	-	+	+	-	+
39 *Fusarium pallindoroseum* (6154)	+	-	+	+	-	+	+	-	+	-	-	-
40 *Fusarium reticulatum* (6106)	+	-	+	+	-	+	+	-	+	-	-	-
41 *Fusarium sambucinum* (6156)	+	-	+	+	-	+	+	-	+	-	-	-
42 *Fusarium solani* (6326)	+	-	+	+	-	+	+	-	+	-	-	-
43 *Fusarium sporotrichioides* (6151)	+	-	+	+	-	+	+	-	+	-	-	-
44 *Fusarium tricinctum* (6155)	+	-	+	+	-	+	+	-	+	+	-	+
45 *Fusarium verticillioides* (6065)	+	-	+	+	-	-	-	-	-	-	-	-
46 *Absidia coerulea* (6900)	-	-	-	-	-	-	-	-	-	-	-	-
47 *Alternaria alternata* (6005)	-	-	-	-	-	-	-	-	-	-	-	-
48 *Alternaria mali* (6972)	-	-	-	-	-	-	-	-	-	-	-	-
49 *Candida albicans* (7965)	-	-	+	-	-	-	-	-	-	-	-	-
50 *Candida solani* (7185)	-	-	-	-	-	-	-	-	-	-	-	-
51 *Cladosporium cladosporioides* (6167)	-	-	-	-	-	-	-	-	-	-	-	-
52 *Cladosporium resinae* (6019)	+	+	-	+	+	-	+	+	-	+	+	-
53 *Geotrichum candidum* (6195)	-	-	-	-	-	-	-	-	-	-	-	-
54 *Geotrichum fragrans* (6186)	-	-	-	-	-	-	-	-	-	-	-	-
55 *Mucor circinelloides* (6164)	-	-	-	-	-	-	-	-	-	-	-	-
56 *Mucor racemosus* (6119)	-	-	-	-	-	-	-	-	-	-	-	-
57 *Rhizopus oligosporus* (6969)	-	-	-	-	-	-	-	-	-	-	-	-
58 *Rhizopus oryzae* (6945)	-	-	-	-	-	-	-	-	-	-	-	-
59 *Trichoderma viride* (6951)	+	+	+	+	-	+	-	-	+	-	-	-

[1]Ab-mix sELISA was done according to the procedure described elsewhere in this paper.

[2]KCTC number.

[3]Dilution ratio of fungal culture broth.

[4]Reactivity of each culture broth towards Ab-mix ELISA using MAb 1G11 and 1B8 was scored as follows; (-) A_{450}<5% Positive control, (+) 5% positive control<A_{450}. Positive control: *F. moniliforme* EPS 10 μg/ml for MAb 1B8, *P. citrinum* EPS 10 μg/ml for MAb 1G11, negative control: PBST buffer.

[5]Reactivity of each culture broth towards sandwich ELISA using MAb 1G11 alone in previous experiment [9].

[6]Reactivity of each culture broth towards sandwich ELISA using MAb 1B8 alone in previous experiment [10].

foods, using polyclonal antibody against a mixture of six common molds; *A. versicolor, Cladosporium berbarum, F. poae, Geotrichum candidum, Mucor circinelloides*, and *P. chrysogenum*. This sandwich ELISA system could detect these molds in addition to 10 other genera, but not yeast. However, they used only one polyclonal antibody. On the other hand, Wyatt *et al.* [20] could detect different serotypes of *Salmonella typhimurium*, using a cocktail of monoclonal Abs as a detector stage in sandwich ELISA. Their ELISA format was different from that of Yong and Cousin [21], and Wyatt *et al.* [20] used a mixture of MAbs for the detecting step of sandwich ELISA. Furthermore, these results were limited to bacteria, but not molds.

In conclusion, the Ab-mix sELISA developed in this study showed that different MAbs for mold could be used for Ab-mix sELISA for simultaneously detecting three species of the molds, *Aspergillus*, *Penicillium*, or *Fusarium*, using one ELISA system.

Acknowledgments

This study was supported by grants from the National Agricultural Cooperative Federation and from the Korea Health Ministry of Health and Welfare (HMP-98-F-2-004), Republic of Korea.

References

1. Bullerman, L. B. 1986. Mycotoxins and food safety. *Food Technol.* **May:** 59-66.
2. Chen, G. C. and B. R. Johnson. 1983. Improved colorimetric determination of cell wall chitin in wood decay fungi. *Appl. Environ. Microbiol.* **46:** 13-16.
3. Cousin, M. A., S. S. Zeidler, and P. E. Nelson. 1984. Chemical detection of mold in processed foods. *J. Food Sci.* **49:** 439-445.

4. De Ruiter, G. A., S. H. W. Notermans, and F. M. Rombouts. 1993. New methods in food mycology. *Trends Food Sci. Technol.* **4:** 91-97.
5. Donald, W. W. and C. J. Microcha. 1977. Chitin as a measure of fungal growth in stored corn and soybean seed. *Cereal Chem.* **54:** 466-474.
6. Jarvis, B. 1977. Chemical method for the estimation of mould in tomato products. *J. Food Technol.* **12:** 581-591.
7. Jarvis, B., D. A. L. Seiler, A. L. Ould, and A. P. Williams. 1983. Observations on the enumeration of moulds in food and feedingstuffs. *J. Appl. Bacteriol.* **55:** 325-336.
8. Kwak, B. Y., S. Y. Kim, and D. H. Shon. 1999. Detection of mold by enzyme-linked immunosorbent assay. *J. Microbiol. Biotechnol.* **9:** 764-772.
9. Kwak, B. Y., D. H., Shon, B. J. Kwon, C. H. Kweon, and K. H. Lee. 2001. Detection of *Aspergillus* and *Penicillium* genera by enzyme-linked immunosorbent assay using a monoclonal antibody. *J. Microbiol. Biotechnol.* **11:** 21-28.
10. Kwak, B. Y., B. J. Kwon, C. H. Kweon, and D. H. Shon. 2003. Detection of *Fusarium* species by enzyme-linked immunosorbent assay using a monoclonal antibody. *J. Microbiol. Biotechnol.* **13:** 794-799.
11. Mousing, J., P. T. Jensen, C. Halgaard, F. Bager, N. Feld, B. Nelsen, J. P. Jielsen, and S. Bech-Nielsen. 1997. Nationwide *Salmonella enterica* surveillance and control in Danish slaughter swine herds. *Prev. Vet. Med.* **29:** 247-261.
12. Nielsen, B., D. Baggesen, F. Bager, J. Haugegaard, and P. Lind. 1995. The serological response to *Salmonella* serovars *typhimurium* and *infantis* in experimentally infected pigs. The time course followed with an indirect anti-LPS ELISA and bacteriological examinations. *Vet. Microbiol.* **47:** 205-218.
13. Lin, H. H. and M. A. Cousin. 1987. Evaluation of enzyme-linked immunosorbent assay for detection of mold in foods. *J. Food Sci.* **52:** 1089-1096.
14. Notermans, S. and C. J. Heuvelman. 1985. Immunological detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); Preparation of antigens. *Int. J. Food Microbiol.* **2:** 247-258.
15. Notermans, S., C. J. Heuvelman, H. P. van Egmond, W. E. Paulsch, and J. R. Besling. 1986. Detection of mold in food by enzyme-linked immunosorbent assay. *J. Food Protect.* **49:** 786-791.
16. Notermans, S., G. Wieten, H. W. B. Engel, F. M. Rombouts, P. Hoogerhout, and J. H. van Boom. 1987. Purification and properties of extracellular polysaccharide (EPS) antigens produced by different mould species. *J. Appl. Bacteriol.* **62:** 157-166.
17. Pitt, J. I. and A. D. Hocking. 1997. *In* Pitt, J. I. and Hocking, A. D. (eds), *Fungi and Food Spoilage*. Blackie Academic & Professional, London.
18. Roberston, A. and N. Patel. 1989. Development of an enzyme immunoassay for the quantification of mould in tomato paste-an alternative to the Howard mould count. *Food Agric. Immunol.* **1:** 73-81.
19. Seitz, L. M., H. E. Mohr, R. Burroughs, and D. B. Sauer. 1977. Ergosterol as an indicator of fungal invasion in grains. *Cereal. Chem.* **54:** 1207-1217.
20. Wyatt, G. M., M. N. Langley, H. A. Lee, and M. R. A. Morgan. 1993. Further studies on the feasibility of one-day *Salmonella* detection by enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* **59:** 1383-1390.
21. Yong, R. K. and M. A. Cousin. 1995. Nonspecific enzyme-linked immunosorbent assay for mold in foods. *J. Food Sci.* **60:** 1357-1363.