Diagnosis of Systemic Candidiasis by an Enzyme-Linked Dot Immunobinding Assay for a Circulating Immunodominant 47-Kilodalton Antigen

RUTH MATTHEWS* AND JAMES BURNIE

Department of Medical Microbiology, St. Bartholomew's Hospital, London EC1A 7BE, United Kingdom

Received 19 August 1987/Accepted 20 November 1987

A dot immunobinding assay based on the detection of the immunodominant 47-kilodalton (kDa) antigen of *Candida albicans* is described for the serological diagnosis of systemic candidiasis. It was compared with a reverse passive latex agglutination test and a dot immunobinding assay with total unfractionated hyperimmune serum to *C. albicans*. Use of the 47-kDa antigen-specific probe increased both the sensitivity and specificity of the assay system. Patients with systemic candidiasis were detected earlier in the course of the infection. The rate of detection of systemic *C. albicans* infections in neutropenic patients was 77% compared with 55% with total antibody in the dot immunobinding assay and 29% with the latex test. All three assay systems were positive in over 73% of infected patients who were not neutropenic. The 47-kDa antigen-specific probe was relatively specific to *C. albicans*. Antibody probes to the immunodominant antigens of other yeasts might be incorporated in the same dot immunobinding assay to detect systemic candidiasis caused by other species of yeasts.

In most patients, systemic candidiasis produces few distinctive clinical signs (7), and several series have shown that blood cultures are positive in only a minority of cases (5). The treatment of choice, amphotericin B, is associated with pronounced nephrotoxicity which has made clinicians reluctant to use it without a firm diagnosis (15). This, together with the rising incidence (12), has led to many attempts to produce reliable serological test for systemic *Candida* infection. The detection of serum antibody does not distinguish systemic candidiasis from superficial infection and mucosal colonization and is probably of more value prognostically than diagnostically (10). *Candida albicans* is a common commensal, and many healthy individuals have low titers of specific antibody (Editorial, Lancet ii:1373-1374, 1986). Conversely, high-risk immunocompromised patients often produce little antibody during systemic infections. There is therefore an urgent need for a sensitive, specific test based on antigen detection.

Reverse passive latex agglutination (RPLA) has become one of the most widely used antigen detection systems for the serodiagnosis of systemic candidiasis. Latex particles have been coated with candidal antiserum raised in rabbits against whole candidal cells (2, 6, 9) or cytoplasmic extracts (3). These tests are quick and simple to perform, and some are now available commercially but have been criticized for lack of sensitivity and specificity (2, 3). The latter is particularly a problem in patients with autoantibodies, and sensitivity is poorer in neutropenic than in nonhematological patients (11). The number and nature of the antigens being detected by these latex tests is unknown.

We previously described the serological response to systemic candidiasis by immunoblotting serial sera from infected patients (10, 11). This demonstrated the immunodominance of an antigen of 47 kilodaltons (kDa), with 74% of patients (68 of 93) producing antibody to this component (11). Similar immunodominant antigens have been described by Strockbine et al. (44 to 52 kDa) (16), Greenfield and Jones (48.9 to 59.7 kDa) (8), and Au-Young et al. (45 kDa) (1). It is

likely that these antigens are equivalent or closely related, with the differences in molecular weight being due to variation in methods of antigen preparation and antibody detection. In each case, antibody to this antigen was produced by the majority (>70%) of patients but was much less common in superficially infected or uninfected controls. We isolated this antigen from the sera of patients with systemic candidiasis by affinity chromatography (11). Neale et al. (13) demonstrated the 47-kDa antigen in circulating immune complexes in a patient with *C. albicans* osteomyelitis. Since it appears to be specifically liberated in invasive disease it is an obvious candidate for the development of a new generation of serodiagnostic tests for systemic candidiasis.

To detect circulating 47-kDa antigen in sera from patients, we applied a new dot immunobinding system. This system is much more sensitive than latex agglutination and provides a permanent visual record, and results are available within 24 h (17). A major problem with dot immunobinding systems can be the high background levels of nonspecific binding, but this was overcome by affinity purification of the anticandidal rabbit antiserum to provide a monospecific antibody probe against the 47-kDa antigen. This paper describes the use of this new assay system in the serodiagnosis of systemic candidiasis.

MATERIALS AND METHODS

Patients. Sera were obtained from 35 neutropenic patients with lymphoproliferative disorders and 52 nonneutropenic medical and surgical patients with proven invasive candidiasis caused by *C. albicans*. Invasive candidiasis was defined either by two or more sets of positive blood cultures taken from separate intravenous sites at least 24 h apart or by histological and culture evidence obtained at postmortem. Serial sera (average number, 7; range, 2 to 15) taken at least 2 days apart were available from 46 of these patients. Sera were examined from a further 18 patients with invasive disease caused by *Candida tropicalis* ($n = 4$), *Candida parapsilosis* ($n = 6$), *Trichosporon beigelii* ($n = 1$), *Blastoschizomyces capitatus* ($n = 3$), and *Torulopsis glabrata* ($n =$

* Corresponding author.

4). Sera were also obtained from 9 nonneutropenic patients with candidal urinary tract infections, 18 with oral candidiasis, 3 with a colonized wound, and 2 with vaginal candidiasis. A further 75 neutropenic and 50 nonneutropenic patients with no evidence of infection or colonization caused by *C. albicans* provided the control population. In all, a total of 550 sera were assayed, of which 393 were from the systemic cases.

Preparation of rabbit candidal antisera. A pressate of *C. albicans* NCTC 3153 serotype A was prepared with an Xpress (LKB, Bromma, Sweden) at a pressure of 200 mPa at -20°C . A 25-mg portion of this pressate was mixed with 0.2 ml of Freund complete adjuvant and injected subcutaneously into three New Zealand White rabbits. A further injection was given at 14 days. Serum obtained at 28 days was fractionated to produce monospecific antibody to the 47-kDa antigen by a modification of the technique of Olmsted (14). The candidal pressate was heated with cracking buffer (2.6% sodium dodecyl sulfate, 1.3% 2-mercaptoethanol, 6% glycerol, 0.2% bromophenol blue, 0.05 M Tris hydrochloride [pH 6.8]) at 100°C for 5 min before being loaded onto a 10% polyacrylamide gel as previously described (11). After electrophoresis of the candidal pressate and immunoblotting onto nitrocellulose, the horizontal band containing the 47-kDa antigen was excised. Free protein-binding sites were blocked with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline, pH 7.5. After being washed in phosphate-buffered saline, the strip was then incubated for 2 h with the rabbit hyperimmune anticandidal serum. The strip was washed again before the bound antibody was eluted with glycine buffer (0.2 M glycine, 1 mM EGTA, pH 2.7), which was then neutralized with an equal volume of 100 mM Tris base. The specificity of the affinity-purified anti-47-kDa antigen probe was confirmed by immunoblotting against the pressate of *C. albicans*.

Dot immunobinding assay. The total unfractionated hyperimmune serum and the affinity-purified anti-47-kDa antigen antiserum were both used as probes in the dot immunobinding assay. A 100- μl sample of serum from each patient was loaded onto nitrocellulose in the dot immunobinding plate (Bio-Rad Laboratories, Richmond, Calif.) and allowed to drain through the nitrocellulose membrane under gravity control. This was followed by 100 μl of Tris-buffered saline (pH 7.5) and then by 200 μl of Tris-buffered saline–0.05% Tween 80 under suction. The membrane was subsequently removed and blocked overnight at 4°C in 3% bovine serum albumin in Tris-buffered saline to remove free protein-binding sites. The membrane was then incubated for 2 h in 30 ml of 3% bovine serum albumin in Tris-buffered saline containing 30 μl of affinity-purified anti-47-kDa antigen antibody or unfractionated hyperimmune antiserum. After being washed in 0.9% (wt/vol) saline–0.05% Tween 80 five times over 30 min, the membrane was incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). The conjugate was diluted 1 in 1,000 immediately before use in 3% bovine serum albumin in Tris-buffered saline. After being washed as above, membranes were incubated in a freshly prepared and filtered mixture of equal volumes of naphthol ASMX phosphate (Sigma; 0.4 mg/ml in distilled water) and fast red TR salt (Sigma; 6 mg/ml in 0.2 M Tris [pH 8.2]) as previously described (11). A positive control of candidal pressate (4 $\mu\text{g}/\text{ml}$) was included with each batch of test sera as were known negative sera as negative controls. Antigen-antibody binding was identified by the deposition of red stain and quantified by densitometry (Chro-

FIG. 1. Reactions of the 47-kDa antigen-specific antibody probe with sera from systemically infected and colonized patients. When multiple sera were available from a patient, the maximum antigen titer was taken. Reactions are graded as nil (<250 units), trace (250 to 350 units), positive (350 to 500 units), and strongly positive (>500 units).

moscan 3; Joyce Loebl; kindly provided by the Virology Department, St. Bartholomew's Hospital).

RPLA. Latex was sensitized with the rabbit hyperimmune candidal antiserum as described elsewhere (3). Serum (30 μl) from a patient was mixed with 30 μl of sensitized latex particles on a clean glass slide. The slide was rotated manually for 2 min at room temperature. The titers of positive sera were determined by the serial dilution of sera in glycine-buffered saline (pH 8.4).

RESULTS

Calibration of dot immunobinding assay. Serial dilution of the *C. albicans* pressate probed with the 47-kDa antigen-specific antibody demonstrated that the intensity of the dot as determined by densitometry was proportional to the amount of antigen present. As little as 0.4 μg of total candidal protein per ml was detectable. The reactions with sera from patients were graded as negative (<250 units), trace (250 to 350 units), positive (350 to 500 units), and strongly positive (>500 units).

Patients with systemic candidiasis. The distribution of the antigen titers obtained in each assay system for sera from each of the 105 systemically infected patients is illustrated by Fig. 1 (47-kDa antigen-specific antibody probe), 2 (total unfractionated antibody), and 3 (RPLA). Positive results were obtained from 76.2% of systemically infected patients with the 47-kDa antigen-specific antibody probe in the dot immunobinding assay, compared with 69.6% with unfractionated hyperimmune serum and only 56.2% by RPLA (Table 1). When patients infected with species other than *C. albicans* were excluded, the percent positive results with the 47-kDa antigen-specific antibody improved to 83.9%, whereas results remained within 2% of their previous values with total antibody in the dot immunobinding assay and the

FIG. 2. Reactions of the unfractionated hyperimmune rabbit candidal antiserum with sera from systemically infected and colonized patients. Reactions are graded as described in the legend to Fig. 1.

RPLA. The 47-kDa antigen-specific antibody probe was most suitable for detecting systemic *C. albicans* infections and relatively insensitive at detecting systemic infections caused by species other than *C. albicans* (Table 2). Assays with total unfractionated hyperimmune serum, either in the dot immunobinding system or by RPLA, were better at detecting these infections. Detection was highest for *C. parapsilosis* infections, with all six patients giving positive results with the unfractionated serum, and poorest for patients with *T. glabrata* infections, with none of the four patients giving positive results.

Greater than 73% of nonneutropenic systemically infected patients gave positive results for all three assay systems (Table 3). In the neutropenic group, detection with total

FIG. 3. Antigen titers of sera from systemically infected and colonized patients was determined by RPLA with particles sensitized with unfractionated hyperimmune rabbit candidal antiserum.

TABLE 1. Results of patients with systemic *C. albicans* and other yeast infections^a

Infection and test	No. of cases	% True positive	% Weak positive	% False negative
Systemic yeast	105			
Dot immunobinding				
Total antibody		69.6	8.5	21.9
47-kDa antigen specific		76.2	16.2	7.6
RPLA		56.2	19.0	24.8
Systemic <i>C. albicans</i>	87			
Dot immunobinding				
Total antibody		67.8	10.4	21.8
47-kDa antigen specific		83.9	13.8	2.3
RPLA		55.2	23.0	21.8

^a Negative: Densitometry, <250 units; RPLA titer, ≤4; weak positive: densitometry, 250 to 350 units; RPLA titer, 8; positive: densitometry, >350 units; RPLA titer, ≥10.

unfractionated antibody was much poorer, with 54.8% being positive in the immunobinding assay and only 29% positive by the RPLA. Use of the 47-kDa antigen-specific antibody greatly increased the sensitivity of the dot immunobinding assay for neutropenic patients, with 77.4% giving positive results.

The 47-kDa antigen-specific antibody probe also compared favorably with total antibody in its ability to detect systemic candidiasis earlier (Table 4). The initial serum, taken when the referring clinician first considered that systemic candidiasis was a likely diagnosis clinically, was positive in only 21.7% (10 of 46 patients) by the RPLA compared with 34.8% positive results with total antibody in the dot immunobinding assay and 50% positive results with the 47-kDa antigen-specific antibody. Subsequent follow-up sera became positive in 50% of patients by the RPLA, in 74% with total antibody in the dot immunobinding assay, and in 89.1% with 47-kDa antigen-specific antibody.

Colonized and superficially infected patients. The ability of the three assay systems to distinguish colonized from systemically infected patients is shown in Fig. 1, 2, and 3. Of these 32 patients, 1 gave a positive result in both of the dot immunobinding systems, and this patient also had the highest RPLA titer of this group, which was a borderline-positive titer of 8. This patient had a candidal urinary tract infection

TABLE 2. Results of the 18 patients with systemic infection caused by yeasts other than *C. albicans*

Infection (no. of patients)	Dot immunobinding (no. of patients) ^a		Mean RPLA titer (range)
	Total antibody	47-kDa antigen specific	
<i>C. parapsilosis</i> (6)	+ (4) ++ (2)	+ (4) ± (1) 0 (1)	20 (10–40)
<i>C. tropicalis</i> (4)	+ (4)	± (3) + (1)	10 (2–20)
<i>T. glabrata</i> (4)	0 (4)	0 (4)	0
<i>B. capitatus</i> (3)	++ (2) + (1)	++ (1) + (1) ± (1)	20 (4–40)
<i>T. beigeli</i> (1)	++ (1)	0 (1)	40

^a 0, nil (<250 units); ±, trace (250 to 350 units); +, positive (350 to 500 units); ++, strong positive (>500 units).

TABLE 3. Results of the 87 patients with systemic candidiasis caused by *C. albicans* categorized into neutropenic and nonneutropenic patients^a

Category and test	No. of cases	% True positive	% Weak positive	% False negative
Neutropenic	31			
Dot immunobinding				
Total antibody		54.8	16.2	29
47-kDa antigen specific		77.4	19.3	3.3
RPLA		29.0	29.0	42.9
Nonneutropenic	56			
Dot immunobinding				
Total antibody		78.6	7.1	14.3
47-kDa antigen specific		87.5	12.5	0
RPLA		73.3	19.6	7.1

^a Positive, weak positive, and negative are as defined in Table 1, footnote a.

which responded to 5-fluorocytosine. All other patients in this group had negative RPLA tests (titer of ≤ 4). With the dot immunobinding assays, one in four gave trace positive results and none gave strong positive results.

Control patients. When the dot immunobinding assay was applied to the 125 control patients, negative results were obtained with 92.8% of sera probed with the 47-kDa antigen-specific antibody and 72% of sera with the total unfractionated antibody. Five sera gave a positive reaction with both antibody probes, and these sera were all hemolyzed. Trace positive reactions were found in 3.2% of sera with the 47-kDa antigen-specific antibody. Two of these patients had severe jaundice, and two had invasive aspergillosis. The total antibody probe produced trace positive results in 24% of sera, and this reflected an overall higher background with the unfractionated antiserum.

The RPLA was negative in 79.2% of sera and produced a titer of 1 in 2 in 12.8% of sera and a titer of 1 in 4 or greater in 8%. Eight of these latter sera were strongly rheumatoid factor positive, and two occurred in patients with septicemias caused by group JK corynebacteria. The two patients with JK septicemias also gave a positive result with total antibody in the dot immunobinding assay but not with the 47-kDa antigen-specific antibody.

DISCUSSION

The development of a serodiagnostic test for systemic candidiasis based on detection of the circulating immunodominant 47-kDa antigen increased both its sensitivity and specificity for disseminated *C. albicans* infections. This increased sensitivity was useful in detecting cases of systemic candidiasis earlier in the course of the infection. A total of 50% of the first sera sent from patients with systemic candidiasis were positive with the 47-kDa antigen-specific antibody compared with 34.8% with total antibody in the dot immunobinding assay and 21.7% by the RPLA. Neutropenic patients particularly benefited from the greater sensitivity of the assay system because latex agglutination gives poor results in this group, possibly because they often produce immunoglobulin M, which has a higher avidity for antigens than immunoglobulin G (11). The rate of detection of systemic *C. albicans* infections in neutropenic patients rose from 29% by the RPLA to 77% with the 47-kDa antigen-specific antibody probe in conjunction with the dot immunobinding assay. In nonneutropenic patients, the detection

rate rose from 73% by RPLA to 87% with the 47-kDa antigen-specific antibody.

The dot immunobinding assay overcomes many of the problems inherent in latex agglutination. It circumvents the common problem of nonspecific agglutination resulting from autoantibodies and provides a permanent visual record which can be objectively read by densitometry. False-positive results occurred to a titer of 1 in 4 or greater in 8% of the 125 negative control sera by the RPLA. Weak false-positive results were frequent (28%) with total antibody in the dot immunobinding assay, but this high level of background reactivity was reduced to 3% false-positive results by using the specific fractionated 47-kDa antigen-specific antibody. The number of false-positive results could be further reduced by taking care not to hemolyze specimens since hemolysis was the commonest cause of false-positive results. Sera from jaundiced patients could give falsely high readings on the densitometer, but their yellow color was readily distinguished by eye from the red of a true positive result. Two patients with septicemias caused by group JK corynebacteria gave false-positive reactions by both RPLA and the dot immunobinding assay with total antibody but were negative with the 47-kDa antigen-specific antibody probe. This may be due to cross-reaction between the galactomannan present in the cell walls of JK corynebacteria and the mannan in *C. albicans* cell walls.

Colonized and superficially infected patients produced negative or weakly positive results in all three assay systems, with the exception of one patient with a candidal urinary tract infection (Fig. 1 to 3). The 47-kDa antigen-specific immunoassay showed the greatest ability to discriminate between systemically infected patients and patients who were colonized or superficially infected, with only 23.8% of patients with systemic candidiasis giving negative or weak positive results. The RPLA was the poorest assay system, with 43.8% of systemic cases giving negative or weak positive results (Fig. 1 to 3; Table 1). In other series, latex tests have been reported to detect between 28 and 90% of cases of systemic candidiasis, with the percent positive being lowest in neutropenic patients (2, 6).

The 47-kDa antigen-specific probe was relatively poor at detecting systemic candidiasis caused by *Candida* species other than *C. albicans*. At present, this represents a small minority of cases in the United Kingdom. Nevertheless, it

TABLE 4. Results of serial sera from 46 patients with systemic candidiasis caused by *C. albicans*^a

Test	% True positive	% Weak positive	% False negative
Dot immunobinding			
Total antibody			
Initial serum ^b	34.8	13	52.2
Subsequent serum ^b	74	13	13
47-kDa antigen specific			
Initial serum	50	32.6	17.4
Subsequent serum	89.1	10.9	0
RPLA			
Initial serum	21.7	10.9	67.4
Subsequent serum	50	21.7	28.3

^a Negative, densitometry, <250 units; RPLA titer, ≤ 4 ; weak positive, densitometry, 250 to 350 units; RPLA titer, 8; positive, densitometry, >350 units; RPLA titer, ≥ 10 .

^b Initial serum was serum sent to laboratory when clinician first considered that systemic candidiasis was a likely diagnosis clinically; subsequent serum was the serum showing the maximum antigen titer in subsequent follow-up sera.

should be possible to incorporate antibody probes to immunodominant antigens from other yeast species into the dot immunobinding assay to increase its spectrum of reactivity. When total unfractionated hyperimmune serum to *C. albicans* was used, all six patients with *C. parapsilosis* infections gave positive results by both the dot immunobinding assay and the RPLA. This reflects the high degree of cross-reactivity between *C. albicans* and *C. parapsilosis*, previously demonstrated by immunoblotting, whereas cross-reactivity with *C. tropicalis* and *T. glabrata* is less (4).

Our results suggest that a latex agglutination test, some of which are available commercially, is best used as a screening test because it is quick and simple to perform on individual sera. It will give a high percentage of false-negative results in neutropenic patients and some false-positive results owing to autoantibodies. The dot immunobinding assay may be used to overcome this, but takes 24 h to give a result and is best used to test batches of sera, as would be available in a referral center. In patients with systemic *C. albicans* infections, the use of an antibody probe specific to the 47-kDa component increases both sensitivity and specificity. The increased sensitivity is particularly necessary in neutropenic patients, for whom other diagnostic tests have proved inadequate (Editorial, *Lancet* ii:1373–1374, 1986). At present, *C. albicans* is the predominant cause of systemic candidiasis, but if this were to change, antibody probes to immunodominant components of other yeasts could readily be incorporated in the dot immunobinding assay in the form of a cocktail of monoclonal antibodies.

ACKNOWLEDGMENTS

R. C. Matthews is a Wellcome Trust senior research fellow.

We are grateful to the following hospitals for sending us sera: Derriford Hospital, Plymouth; St. Bartholomew's Hospital, London; The London Hospital; The Homerton Hospital, London; The Harley Street Clinic, London; The London Clinic; The Christie Hospital, Manchester; The Withington Hospital, Manchester.

LITERATURE CITED

1. Au-Young, J. K., F. A. Troy, and E. Goldstein. 1985. Serologic analysis of antigen-specific reactivity in patients with systemic candidiasis. *Diagn. Microbiol. Infect. Dis.* 3:419–432.
2. Bailey, J. W., E. Sada, C. Brass, and J. E. Bennett. 1985. Diagnosis of systemic candidiasis by latex agglutination for serum antigen. *J. Clin. Microbiol.* 21:749–752.
3. Burnie, J. P. 1985. A reverse passive latex agglutination for the diagnosis of invasive candidosis. *J. Immunol. Methods* 82:267–281.
4. Burnie, J. P., R. C. Matthews, A. Fox, and S. Tabaqchali. 1985. Use of immunoblotting to identify antigenic differences between the yeast and mycelial phases of *Candida albicans*. *J. Clin. Pathol.* 38:701–706.
5. de Repentigny, L., and E. Reiss. 1984. Current trends in immunodiagnosis of candidosis and aspergillosis. *Rev. Infect. Dis.* 6:301–312.
6. Gentry, L. O., I. D. Wilkinson, A. S. Lea, and M. F. Price. 1983. Latex agglutination test for detection of candidal antigen in patients with disseminated disease. *Eur. J. Clin. Microbiol.* 2:122–128.
7. Gold, J. W. M. 1984. Opportunistic fungal infections in patients with neoplastic disease. *Am. J. Med.* 76:458–463.
8. Greenfield, R. A., and J. M. Jones. 1981. Purification and characterization of a major cytoplasmic antigen of *Candida albicans*. *Infect. Immun.* 34:469–477.
9. Kahn, F. W., and J. M. Jones. 1986. Latex agglutination test for detection of *Candida* antigens in sera of patients with invasive candidiasis. *J. Infect. Dis.* 153:579–585.
10. Matthews, R. C., J. P. Burnie, and S. Tabaqchali. 1984. Immunoblot analysis of the serological response in systemic candidosis. *Lancet* ii:1415–1418.
11. Matthews, R. C., J. P. Burnie, and S. Tabaqchali. 1987. Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to *Candida albicans*. *J. Clin. Microbiol.* 25:230–237.
12. Meunier-Carpentier, F., T. E. Kiehn, and D. Armstrong. 1981. Fungaemia in the immunocompromised host. Changing patterns, antigenaemia, high mortality. *Am. J. Med.* 71:363–370.
13. Neale, T. J., J. C. Muir, and B. Drake. 1987. The immunochemical characterisation of circulating immune complex constituents in *Candida albicans* osteomyelitis by isoelectric focusing, immunoblot and immunoprint. *Aust. N.Z. J. Med.* 17:201–209.
14. Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.
15. Seelig, M. S., and P. J. Kozinn. 1982. Clinical manifestations and management of candidosis in the compromised patient, p. 49–92. In D. W. Warnock and M. D. Richardson (ed.), *Fungal infection in the compromised patient*. John Wiley & Sons, Inc., New York.
16. Strockbine, N. A., M. T. Largen, S. M. Zweibel, and H. R. Buckley. 1984. Identification and molecular weight characterization of antigens from *Candida albicans* that are recognized by human sera. *Infect. Immun.* 43:715–721.
17. Towbin, H., and J. Gordon. 1984. Immunoblotting and dot-immunobinding—current status and outlook. *J. Immunol. Methods* 72:313–340.