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Identification of screwworms, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), with a monoclonal antibody-based enzyme-linked immunosorbent assay (MAb-ELISA)

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Abstract

Myiasis caused by screwworms, *Cochliomyia hominivorax* (Coquerel), is devastating to warm-blooded animals and economically important to livestock producers. It is difficult to distinguish these pests, immature screwworms, from immatures of other non-pest fly species that often occur in animal wounds; it would be helpful to have tools available that do not rely on morphological characteristics. We developed two monoclonal antibodies (MAbs), highly specific for the screwworm, and used them in an enzyme-linked immunosorbent assay (MAb-ELISA), that differentiated screwworm eggs, larvae, pupae, and adults from those of the closely related secondary screwworm, *C. macellaria* (Fabricius) as well as *Phormia regina* (Meigen), *Phaenicia sericata* (Meigen), *Calliphora vicina* Robineau-Desvoidy, and *Chrysomya rufifacies* (Macquart). In a blind study, the microplate MAb-ELISA, which took about 4 h to complete, displayed high specificity (99%), sensitivity (92%) and overall accuracy (97%) in distinguishing all life stages of the screwworm. Electrophoresis results suggested that the two monoclonal antibodies recognized identical conformational epitopes present in all screwworm life stages. The screwworm eradication program, successful in eradicating this pest from the US, Mexico, most of Central America and Libya (after an accidental introduction), could benefit in future eradication, surveillance, and exclusion efforts by developing a reliable field identification kit based on MAb-ELISA that accurately and quickly distinguished cases of screwworm myiasis. Published by Elsevier Science B.V.

Keywords: Monoclonal antibody; ELISA; *Cochliomyia*; Screwworm

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1. Introduction

Screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), larvae are obligate parasites that feed on living tissues of warm-blooded animals, causing the condition called myiasis (Hall and Wall, 1995). Although eradicated in North America and most of Central America (Galvin and Wyss, 1996), screwworms remain a threat to domestic animals, as well as humans, in currently endemic tropical regions of Central America, South America, and the Caribbean. Furthermore, computer simulation models indicate that the *C. hominivorax* could potentially colonize most of the tropical and semi-tropical regions of the world (Sutherst et al., 1989; Mayer et al., 1992). International livestock trade and movement, as well as human travel, have resulted in the introduction of screwworms to non-endemic areas and those that were previously eradicated (Spradberry, 1994). Screwworms were introduced and became established in Libya in 1988 (El Azazy, 1989) presumably by livestock importation from South America (Beesley, 1991); in France from a dog returning from Brazil (Chermette, 1989); and in Australia by a woman who visited Brazil and Argentina (Searson et al., 1992). In Mexico, which was declared screwworm-free in 1991, an outbreak (69 cases) occurred in 1992 and 1993, about 200 miles from the nearest US border (Galvin and Wyss, 1996). Similarly in the US, several screwworm reintroductions have been reported since its complete eradication in the 1980s (Mehr et al., 1991; Sweis et al., 1997; Garris, 1998; Anon., 1999).

Effective eradication and quarantine measures against screwworms depend on correct species identification. However, early stages of the screwworm are morphologically similar to several species (including the secondary screwworm, *C. macellaria* (Fabricius) and other facultative and scavenger flies in the families Calliphoridae and Sarcophagidae) found in wounds and this has resulted in numerous misidentifications in the past. Recently, molecular biological techniques have provided some accurate means of discriminating insect species, including screwworms. These include both DNA and protein-based identification techniques. Taylor and Peterson (1994) found two allozyme loci diagnostic between *C. hominivorax* and *C. macellaria*. Taylor et al. (1996), and Skoda et al., in press used PCR-RFLP of the mitochondrial DNA (mtDNA) and RAPD-PCR, respectively, to distinguish screwworms from other fly species. But these techniques are limited to the laboratory; requiring specialized equipment and a considerable degree of expertise in following the protocols and interpreting the results. Thus, identification methods that are simple, rapid, accurate, and potentially adaptable for field use are important.

An alternative identification method is the enzyme-linked immunosorbent assay (ELISA) based on specific antibodies against unique screwworm antigens. Figarola (1996) initially developed a blocking ELISA test that used polyclonal antibodies to distinguish the later screwworm instars. However, there was cross-reactivity among other myiasigenic flies and the technique required optimum concentration of blocking antigens to remove non-specific antibodies. In addition, identification of first instars and adults was not possible as the antibodies recognized only larval proteins abundant in second and third instar screwworms.

Development of a simple, more specific monoclonal antibody-based colorimetric assay would be beneficial because of the potential for adaption in an identification kit for screwworm diagnosis usable by most, if not all, individuals. Here, we describe a microplate

ELISA method using screwworm-specific monoclonal antibodies (MAbs) that accurately distinguishes all life stages of the screwworm from other myiasigenic flies.

2. Materials and methods

2.1. Insect samples

Screwworms were obtained from colonies maintained under standard rearing methods, modified from Taylor (1992), at the USDA-ARS Biosecure Screwworm Laboratory in Lincoln, NE. Specimens of *C. macellaria*, *Phormia regina* (Meigen), *Phaenicia sericata* (Meigen), *Calliphora vicina* Robineau-Desvoidy, *Chrysomya rufifacies* (Macquart), and *Sarcophaga* sp. were collected from the vicinity of the laboratory during the summers of 1997–1999 and colonized. Identifications were made by the authors using available keys (Knipling, 1939; Liu and Greenberg, 1989) and confirmed by Woodley (USDA-ARS Systematics Entomology Laboratory, vouchers retained). The non-screwworm adults were kept in separate cages at 25 °C, given water and carbohydrates ad libitum and given daily access to ground beef to lay eggs. Resulting eggs were incubated at 30 °C and, after hatching, the larvae were reared with periodic addition, as needed, of ground beef. After pupation, each species was placed in separate cages where adults were kept similarly to the originally collected females. All specimens from each species were frozen at −70 °C prior to use.

2.2. Antigens and immunizations

All chemicals and other reagents were from Sigma Chem. Co. (St. Louis, MO), except as otherwise noted. First, second, and third instar (25, 10, and 2 individuals, respectively, weighing about 6.4, 7.2, and 10 mg, respectively) *C. hominivorax* were separately homogenized in 0.5 ml of phosphate-buffered saline (PBS), pH 7.4, inside a 1.5 ml microcentrifuge tube using a plastic pestle. The homogenates were centrifuged at 14,000 × g for 2 min to remove the insoluble materials. Protein concentration of the supernatants was determined by the BCA method of Smith et al. (1985) using reagents from Pierce Chemicals (Rockford, IL), with bovine serum albumin as standard. Homogenates of other specimens and other species used in this study followed these same procedures.

Balb/c mice were injected at about 2 week intervals with screwworm antigens over a 2-month period. For the first injection, approximately 100 μg of antigens (33 ug each from first, second and third instars), diluted into 100 μl of PBS and emulsified with an equal volume of complete Freud's adjuvant, were injected intraperitoneally into each animal. For succeeding immunizations, an incomplete adjuvant was used. Intraperitoneal booster injections were made 3 days prior to fusion. This technique was adapted from Harlow and Lane (1988).

2.3. MAb production, screening, and ascites fluid collection

The mice were housed (as directed by UN-L's Animal Care and Use Committee) and monoclonal antibody production was done at the Antibody Core Research Facility, The

Beadle Center for Genetics and Biomaterials Research, University of Nebraska, Lincoln. The general method of Köhler and Milstein (1975) was used for hybridoma production except for the fusion technique. The spleen was removed from the immunized mice, teased apart and cells were fused with non-secreting myeloma cells (sp2/0–Ag14) using high and low molecular weight polyethylene glycol (American Type Culture Collection, Manassas, VA) as outlined in Harlow and Lane (1988). For selection, the hybridomas were resuspended in 200 ml of hypoxanthine-aminopterin-thymidine (HAT) medium, distributed 200 μl per well, and incubated for 3 days at 37 °C and 8% CO_2 in a CO_2 incubator. After 7 days, about half of the HAT media was removed and replaced with hypoxanthine-thymidine (HT) media. The hybridomas were then maintained in vitro in Dulbecco's medium supplemented with 10% horse serum. Approximately 94% fusion was achieved from this method.

Indirect ELISA was used to screen 96 hybridoma culture supernatants for specific MAbs against *C. hominivorax*. Briefly, PVC plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 25 μl of pooled antigen solutions (∼1 μg/μl) in PBS buffer, pH 7.4, of larvae of *C. hominivorax*. The plates were incubated at room temperature for 2 h, and washed three times with ELISA wash solution (0.025% Tween in distilled water). Each well was coated sequentially for 1 h each with 200 ul blocking buffer (5% casein in PBS, 0.001% thimerosal, 0.002% phenol red, pH 7.4), 25 μl undiluted hybridoma culture supernatant, and 25 μl horse radish-peroxidase-conjugated goat antimouse IgG (heavy and light chain; this antibody was used in all succeeding tests; KPL, Gaithersburg, MA) diluted 1:3000 with blocking buffer. Wells were washed between each step three times with ELISA wash solution. Then, 50 μl of ABTS enzyme substrate system (KPL) was added to each well and the plates were incubated for 15 min. Absorbance values at 405 nm were taken using the Biokinetics EL 340 PlateReader (Bio-Tek Instruments Inc., Winoski, VT). Hybridoma supernatants with absorbance values >1.0 were selected for additional screening against pooled samples of larval antigens from *C. hominivorax*, *C. macellaria* and *P. regina* that were prepared using the same methods as above.

From a total of 24 positives against *C. hominivorax*, 2 hybridoma cell MAb-secreting lines (2H11 and 8H3) were chosen for single cell cloning based on very low/absence (<two times the absorbance value, generally <0.15, of negative controls) cross-reactivity with the other fly antigens. Cloning was done twice by limiting dilution (Harlow and Lane, 1988). Cloned hybridoma cells were then injected into mice intraperitoneally to obtain ascites fluid. After 1–2 weeks, ascites fluid was collected from the peritoneal cavity of the mice. The protein concentration of the ascites fluid was determined by BCA method. The ascites fluid was stored in 1 ml aliquots at −70 °C and served as the MAb stock.

2.4. Microtiter plate ELISA

To determine the optimum concentration of ascites fluid, secondary antibody, and antigen for use in subsequent assays, a series of experiments were done that yielded maximum differentiation of specimens through visual assessment. Block titration using indirect ELISA (Figarola, 1996) was performed in these optimization reactions.

In one experiment, separate wells of a PVC plate were coated with 20 μl (about 10 μg of protein) of homogenates from each stage of all seven species and incubated for 1 h

at room temperature. Homogenates for each stage of each species were prepared as in 2.2 above. Then, the plates were washed three times with distilled water to remove unbound antigens before applying 200 μl of blocking buffer to each well for 1 h. After another washing step, 25 μl of 1:1000 dilution of MAb ascites fluid was applied to each well and the plates incubated for 1 h. After unbound MAbs were washed away, the plates were incubated with 25 μl of antimouse conjugate diluted 1:3000 with blocking buffer for another 1 h. The plates were washed three times after incubation, and 50 μl of ABTS enzyme substrate solution was added to each well. After 10 min, absorbance was taken at 405 nm.

In another experiment that was done to mimic field conditions, individuals from all seven species were prepared as follows: first instar = 1 larva in 20 μl PBS, second instar = 1 larva in 100 μl PBS, third instar = 1/2 body in 1000 μl PBS, pupa = 1/2 body in 1000 ul PBS, adult = 1 head in 500 μl PBS. Each sample was homogenized in 1.5 ml microcentrifuge tubes using a disposable plastic pestle (no centrifugation was used). A total of 20 μl of each homogenate was applied to each well, incubated for 1 h at room temperature, and washed three times with wash solution. The concentration and volume of MAb, conjugate, blocking buffer and enzyme substrate, as well as absorbance measurements were as above. For both experiments, four replications were made and the mean absorbance (adjusted with background values) and standard error for each species and stage of insect were calculated. Tukey's test was performed to separate means using commercial software (SAS version 6.1, 1990).

2.5. Blind study

An amount of 115 samples of various stages from each species were used in the blind study. The procedures described above were used to prepare homogenates of individual insects or egg masses (about 5 mg in 500 μl of PBS). The identity and stage of each test sample was sequentially coded and recorded prior to the test. Numbers were then assigned to sample wells, a random number generator used to select a well, and in sequence each sample was assigned to a random position. This was done for two 96-well microplates (Table 1). Fourteen wells of each plate were reserved for two positive controls of each screwworm stage: eggs, first, second and third instars, pupa, adult leg and head. A row of negative control wells, consisting of all solutions except antigens, was also included. The insects were quantitatively scored as "screwworm" or "not screwworm" based on the absorbance values of the test samples (stage known but not the species identity). A sample was classified as screwworm when its absorbance value (multiplied by a factor of 1.5) equaled or exceeded that of the average of the positive control for each stage. The multiplication factor of 1.5 was determined after calculating the average factor of variability between wells containing like known samples, doubling it and then rounding (thus giving a conservative scoring value for the test). Visual, or qualitative, scoring was also done for each test sample by comparing the color of the test samples, or 'unknowns', with that of the respective positive controls. The sensitivity (proportion of screwworms that were identified as screwworms), specificity (proportion of non-screwworm samples identified as non-screwworms) and overall accuracy of the assay (proportion of all samples identified correctly) were calculated.

Table 1
Identities of individual insects used in the microplate ELISA blind study

Insects	Assay well location	
	Plate 1	Plate 2
C. hominivorax		
Egg	C5, D10	B4
First instar	B8, C10, D3, E5, H9	C6, C9, D5, E3, E7, F8, G5
Second instar	B3, F8, G4, G10	C2, F9, H6
Third instar	B6, E7, H7	B7, F6
Pupa	H5	E6
Adult leg	D6, E10, F3	B10, G3
Adult head	D4, E9, H2	B5, E9
C. macellaria		
Egg	B9	
First instar	C3, F6, D8	D6, D11, E4, E8
Second instar	B5, H3	B3, D9, H4, H7
Third instar	B2, C6, F10	C5, F4
Pupa	B8, C4	
Adult leg	D11, G5	C8, G8
Adult head	E3, G8	D4, H9
P. regina		
Egg	B4	
First instar	D7, F5	G11
Second instar	G2	D8, F7
Third instar	D5, E8	C10, G10
Pupa	G6	D10
Adult leg	G9	D2
Adult head	H10	D7
P. sericata		
First instar	C2, C7	D3
Second instar	D2, F7	C7, F3
Third instar	E4, H8	E5, G7
Pupa	G11	
Adult leg	H2	
Adult head	F4	C3
C. vicina		
First instar	B7	F5
Second instar	F2, H6	G2
Third instar	C9	B11
C. rufifacies		
Third instar	C8	H5
Sarcophaga sp.		
First instar	E11, G7	G4
Second instar	B11	E10, G6
Positive controls		
Egg	B1, B12	B1, B12
First instar	C1, C12	C1, C12
Second instar	D1, D12	D1, D12
Third instar	E1, E12	E1, E12
Pupa	F1, F12	F1, F12
Adult leg	G1, G12	G1, G12
Adult head	H1, H12	H1, H12
Negative controls	A1-A12	A1-A12

2.6. Antigen characterization

Homogenates of various stages of *C. hominivorax* were analyzed by Western blotting using both native and sodium-dodecyl sulfate (SDS), polyacrylamide gel electrophoresis (PAGE). Both electrophoreses were performed in a 20 cm Bio-Rad Protean II unit (Bio-Rad Laboratories, Hercules, CA) and followed methods of Sambrook et al. (1989). For native PAGE, 10 μg of insect protein was applied in each lane of a 4–12% linear gradient acrylamide gel with 4% stacking gel (Bio-Rad). For SDS-PAGE, 10 μg of insect protein was applied in each lane of a 4–10% acrylamide stacking and separating gels, respectively. One lane of each gel contained pre-stained molecular weight standards (Bio-Rad). The native gels were run at 20 mA and 4 °C for about 1 h while the SDS gels were run at 200 V for about 45 min.

After electrophoresis, the gels were transferred onto nitrocellulose using a semi-dry electrophoretic transfer apparatus (Bio-Rad Trans-Blot® SD). For both SDS and native PAGE, Towbin's buffer was used (Towbin et al., 1979) except that methanol was removed for native PAGE. After transfer, the membrane was rinsed twice with distilled water. Then, blocking buffer, MAb (diluted 1:1000 with blocking buffer), and goat antimouse conjugate (diluted 1:3000 with blocking buffer) were added sequentially onto the membrane for 1 h each with washing between steps. After the final wash, the membrane was probed with 4-chloro-1-napthol (4CN)/hydrogen peroxide enzyme substrate solution (KPL) and the developing color observed for each lane.

3. Results

Using either pooled homogenates (10 μg) or individual insect samples, both MAbs yielded significantly higher absorbance against *C. hominivorax* than against non-screwworm insects, regardless of which life stage was being assayed (Table 2). All the non-screwworm species tested in this study were non-reactive or had a very low absorbance against the MAbs, including secondary screwworms, *C. macellaria.* Positive screwworm samples were easily identified visually by a green color, while non-screwworms were almost colorless (Fig. 1). Interestingly, the two MAbs had similar absorbance values and color development for each species and stage. We used the same dilution of both antibodies (1:3000 or 0.01 μg/μl) for these microplate assays.

In a blind study using MAb 2H11 and test samples of various stages, the microplate ELISA test displayed 92% sensitivity (36 of 39 screwworm samples were identified correctly), 99% specificity (75 of 76 non-screwworm samples were identified as such) and 97% overall accuracy (111 of 115 specimens were correctly identified) when scored quantitatively (based on absorbance values) (Table 2). The four misidentified samples were: one first instar *C. macellaria* scored as screwworm; two first instars and 1 second instar screwworm scored non-screwworm. Visual scoring of samples produced five additional false negatives (one egg mass, three first instar and 1 second instar of screwworm scored as non-screwworm) (Table 2, Fig. 2), which resulted in a decrease in sensitivity and overall accuracy of the technique (85–94%, respectively). However, for both scoring methods, the scorers, indicating that the MAbs were highly specific to screwworms, identified only one false positive.

Table 2
Mean absorbance values (no correction factor) at 405 nm ± S.E. of homogenates of different fly species and life stages against MAbs 2H11 and 8H3

Species and stage	Mean absorbance[a]			
	Pooled samples[b]		Individual insects[c]	
	2H11	8H3	2H11	8H3
Egg				
C. hominivorax	0.322 ± 0.033 a	0.293 ± 0.018 a	Not tested	Not tested
C. macellaria	0.026 ± 0.005 b	0.039 ± 0.012 b	Not tested	Not tested
P. regina	0.016 ± 0.012 b	0.026 ± 0.008 b	Not tested	Not tested
First instars (<20 h post-oviposition)				
C. hominivorax	0.357 ± 0.029 a	0.385 ± 0.017 a	0.243 ± 0.029 a	0.218 ± 0.033 a
C. macellaria	0.026 ± 0.017 b	0.018 ± 0.007 b	0.013 ± 0.002 b	0.018 ± 0.001 b
P. regina	0.005 ± 0.002 b	0.012 ± 0.002 b	0.010 ± 0.004 b	0.010 ± 0.003 b
P. sericata	0.019 ± 0.011 b	0.017 ± 0.009 b	0.006 ± 0.004 b	0.006 ± 0.004 b
C. vicina	0.007 ± 0.001 b	0.006 ± 0.001 b	0.016 ± 0.005 b	0.010 ± 0.005 b
Sarcophaga sp.	0.014 ± 0.005 b	0.005 ± 0.003 b	0.007 ± 0.003 b	0.028 ± 0.007 b
Second instars (2 days post-oviposition)				
C. hominivorax	0.988 ± 0.027 a	1.029 ± 0.046 a	0.587 ± 0.029 a	0.559 ± 0.045 a
C. macellaria	0.051 ± 0.004 b	0.029 ± 0.006 b	0.038 ± 0.011 b	0.055 ± 0.004 b
P. regina	0.012 ± 0.007 b	0.030 ± 0.008 b	0.019 ± 0.010 b	0.020 ± 0.006 b
P. sericata	0.033 ± 0.007 b	0.028 ± 0.012 b	0.022 ± 0.007 b	0.014 ± 0.002 b
C. rufifacies	0.020 ± 0.006 b	0.013 ± 0.007 b	0.013 ± 0.007 b	0.020 ± 0.008 b
C. vicina	0.011 ± 0.003 b	0.017 ± 0.004 b	0.012 ± 0.006 b	0.022 ± 0.004 b
Sarcophaga sp.	0.015 ± 0.005 b	0.014 ± 0.009 b	0.018 ± 0.002 b	0.022 ± 0.007 b
Third instars (4 days post-oviposition)				
C. hominivorax	1.682 ± 0.023 a	1.832 ± 0.108 a	1.216 ± 0.111 a	1.508 ± 0.095 a
C. macellaria	0.145 ± 0.026 b	0.102 ± 0.011 b	0.090 ± 0.018 b	0.082 ± 0.014 b
P. regina	0.023 ± 0.011 b	0.030 ± 0.007 b	0.037 ± 0.008 b	0.020 ± 0.002 b
P. sericata	0.024 ± 0.016 b	0.048 ± 0.013 b	0.078 ± 0.015 b	0.019 ± 0.004 b
C. rufifacies	0.028 ± 0.006 b	0.029 ± 0.017 b	0.012 ± 0.004 b	0.032 ± 0.007 b
C. vicina	0.017 ± 0.004 b	0.018 ± 0.007 b	0.031 ± 0.004 b	0.033 ± 0.008 b
Pupae				
C. hominivorax	1.885 ± 0.023 a	1.931 ± 0.120 a	1.472 ± 0.100 a	1.569 ± 0.117 a
C. macellaria	0.183 ± 0.007 b	0.124 ± 0.014 b	0.102 ± 0.010 b	0.111 ± 0.009 b
P. regina	0.020 ± 0.011 c	0.023 ± 0.004 b	0.036 ± 0.009 b	0.038 ± 0.006 b
P. sericata	0.016 ± 0.001 c	0.037 ± 0.008 b	0.060 ± 0.015 b	0.028 ± 0.005 b
Adult[c]				
C. hominivorax	1.304 ± 0.069 a	1.455 ± 0.134 a	1.138 ± 0.080(h) a	0.955 ± 0.077(h) a
			1.173 ± 0.097(l) a	1.128 ± 0.085(l) a
C. macellaria	0.084 ± 0.008 b	0.041 ± 0. 020 b	0.063 ± 0.019(h) b	0.044 ± 0.007(h) b
			0.069 ± 0.004(l) b	0.060 ± 0.011(l) b

[a] Four replicates, means having the same letters in each stage are not significantly different (Tukey's Studentized range test, SAS, 1990).
[b] 10 ug of protein applied to each well.
[c] h: head, l: leg.

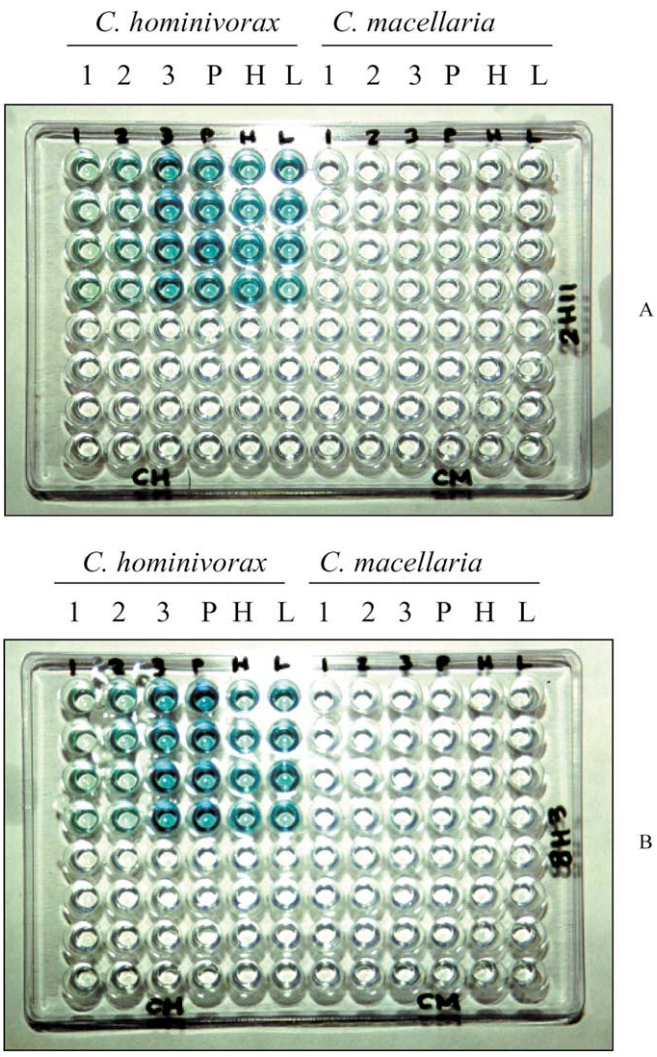

Fig. 1. Microplate ELISA results of *C. hominivorax* (CH) and *C. macellaria* (CM) antigens (individual insects) using MAb 2H11 (A) and MAb 8H3 (B). 1: First instar, 2: second instar, 3: third instar, P: pupa, H: adult head, L: adult leg.

Electrophoresis results showed that both MAbs recognized a similar antigenic component of screwworm proteins present in all stages of the species (Fig. 3). This particular antigenic protein appears to increase in amounts as the insect develops based on the intensity of immunoblot stains seen in each stage. In native gels, this antigenic component has an estimated weight of 190–230 kDa (Fig. 3). However, when the proteins were run on SDS-PAGE, no proteins were detected on immunoblots (data not shown).

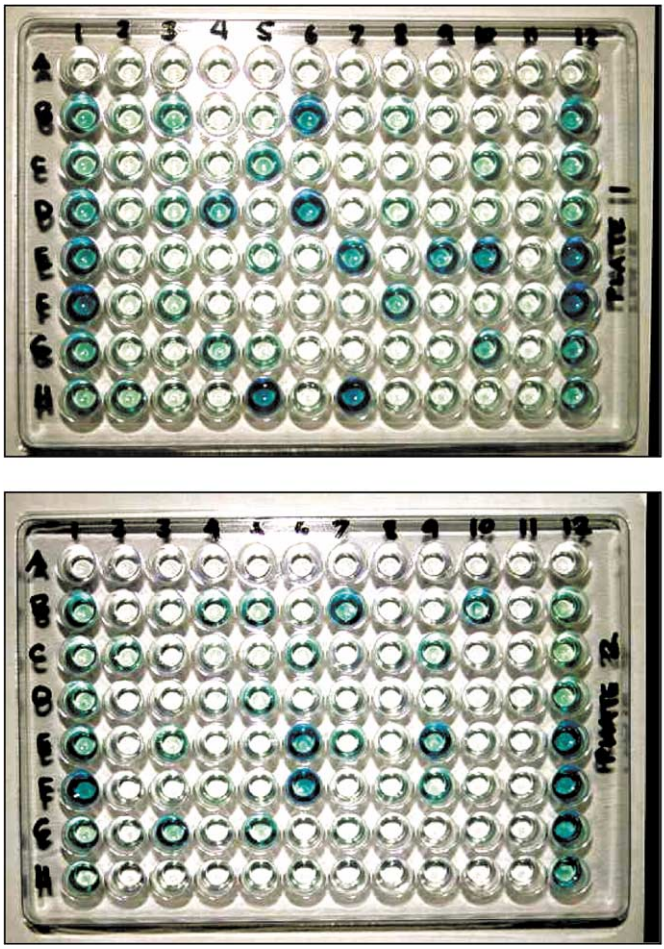

Fig. 2. Visual results of the microplate ELISA blind test using MAb 2H11.

4. Discussion

Although antibodies have been widely used in a variety of applications for the past several years, few studies to date have used these marker molecules to distinguish one insect species from morphologically similar species (Miller, 1981; Ma et al., 1990; Stuart et al., 1994; Zeng et al., 1999). Previously, polyclonal antibodies were used to distinguish screwworms from secondary screwworms in a blocking ELISA format (Figarola, 1996), but cross-reactions were observed among various blow flies to the point that the test was deemed impractical for development of a field identification kit. Yet, the result of cross-reactions to the polyclonal antibodies shows that antigens from all species bound to the microplate wells.

In this work, two screwworm-specific MAbs were developed. These MAbs displayed high sensitivity and specificity against all life stages of the screwworm using the microplate

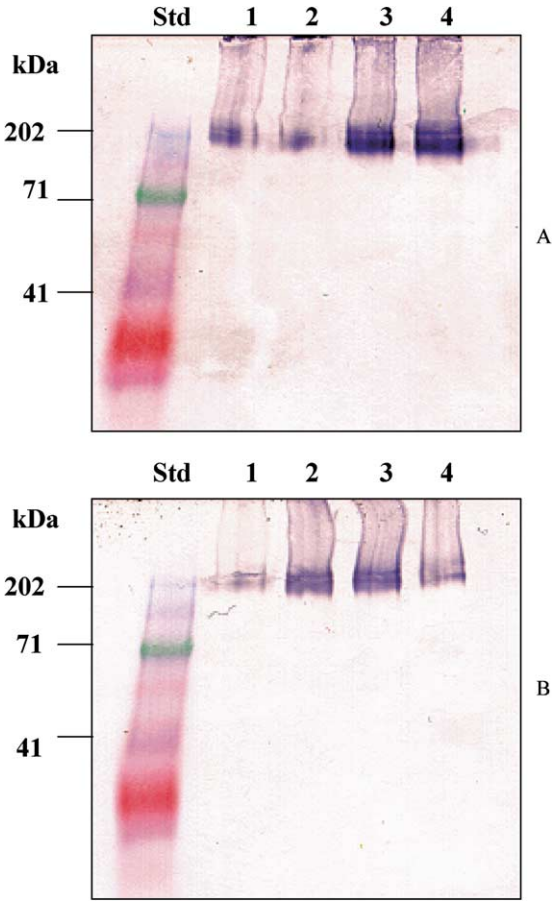

Fig. 3. Homogenates of (1) first instar, (2) second instar, (3) third instar and (4) adults of screwworms subjected to native PAGE and immunoblotted with MAbs 2H11 (A) and 8H3 (B).

ELISA test, and almost no cross-reactions were observed from secondary screwworms and five other myiasigenic flies. Results from our blind tests indicated that this technique was ∼95% accurate for identification of all life stages of the screwworm, even when a single specimen (as in the case of first and second instars), or body parts of a sample were used in the assays. Although there were a few false negatives, particularly when samples were scored visually, only one false positive was recorded for both methods of scoring (75–76 non-screwworms were identified as such). Several of these false negatives could be due to the inherent well-to-well and plate-to-plate variation of the microplates (Burt et al., 1979; Kricka et al., 1980). By multiplying each absorbance value of the test samples by a factor of 1.5 before comparing them with the absorbance values of the positive wells, as done in quantitative scoring, these variations were minimized and resulted in a conservative discrimination of samples. Nevertheless, some of these false negatives could be due to the varying sizes and weights of the screwworm samples used for both pos-

itive controls and the test samples since we assayed individual specimens rather than a known concentration of antigen in an attempt to mimic field conditions. As for the single false positive, it is possible that the particular well was contaminated and/or some of the secondary antibodies were not removed totally during washing. For future development we would replicate the assays of these test samples at least twice in an effort to reduce errant identifications (we only assayed each test sample once per identifier for the blind study).

Scoring microplate ELISA results has traditionally involved arbitrary absorbance cut-off values or setting of statistical confidence limits to the distributions of known positive and negative sample absorbance values because there is always some color development in an ELISA test (Heck et al., 1980; Crowther, 1995). Our cut-off of 1.5 times the absorbance value was not arbitrary, but based on variation detected in these experiments and calculated so to be conservative. Still, as demonstrated in this study, both quantitative and qualitative scoring had similar results in terms of specificity, suggesting that whichever scoring method is used, the chances of false positives are rare. Nonetheless, as indicated above, to further reduce false identifications we suggest that 'unknown' samples be assayed more than once.

Immunoblot results showed that MAbs 2H11 and 8H3 probably reacted with the same native epitopes of the screwworm protein present in all life stages of the insect, but not when these antigenic sites were denatured. Because a particular MAb binds to a unique site (epitope) in the antigen molecule, any disruption or alteration of structure of the antigen will prevent binding of the MAbs to these epitopes (Harlow and Lane, 1988; Berzofsky et al., 1993). As shown in this study, chemical modifications of the native structure of the screwworm antigen by treatment of SDS and mercapto-ethanol prevented any screwworm antigen detection on the surface of nitrocellulose membranes, suggesting that both MAbs failed to bind to denatured proteins and/or probably recognized only conformational epitopes of the screwworm antigen. These results were not surprising since we injected crude homogenates of screwworm proteins to mice during antibody production, and thus, the resulting MAbs only recognized the native antigens and not the denatured state. We also assayed samples (data not presented) that had been preserved in alcohol (the normal storage medium for field collected screwworm samples but which also denatures proteins) and both MAbs failed to react with the screwworm antigens (Figarola, 1999), further supporting our conclusion that these MAbs recognized conformational epitopes.

It is conceivable that this ELISA could be developed into a valuable tool for the rapid field identification of screwworm samples from myiasis wounds. This technique should be useful for the current monitoring, eradication and quarantine efforts against this species. Based on these results, we recommend that samples be assayed immediately, or frozen (at $-70\,^{\circ}C$) indefinitely prior to assay to preserve the native structure of the screwworm antigens and enhance the accuracy of this MAb-ELISA test. This ELISA assay procedure is relatively simple and easy to interpret, but does require laboratory facilities and spectrophotometers for the most accurate results. To circumvent these limitations, we would recommend future development of a quantitative assay (i.e. dot-ELISA) that can be used in the field by most, if not all, individuals of various levels of expertise as a diagnostic kit for screwworm identification. We also recommend that subsamples of all field-collected specimens suspected to be screwworms be preserved in alcohol as vouchers for future reference.

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