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DIFFERENTIATION OF THE SIBLING SPECIES *BIOMPHALARIA OCCIDENTALIS* AND *BIOMPHALARIA TENAGOPHILA* BY THE ELECTROPHORETIC PATTERNS OF THEIR HEMOGLOBIN*

JAMES B. BAILEY**, EDWARD H. MICHELSON** & W. LOBATO PARAENSE***

A simple and rapid method for differentiating the sibling species Biomphalaria tenagophila *and* Biomphalaria occidentalis *by agarose gel electrophoresis (AGE) is described. Snail hemolymph is used as the test sample and the red coloration of the hemoglobin fraction permits visualization of the migration patterns without resorting to specific stains. Moreover, hemolymph samples may be obtained without killing the snail, thus permitting its use for other studies or for breeding.*

Key words: *Biomphalaria occidentalis* – *Biomphalaria tenagophila* – sibling species – hemoglobin electrophoresis – biochemical taxonomy

In 1981, Paraense described *Biomphalaria occidentalis*, a species which could not be differentiated from *Biomphalaria tenagophila* by shell characteristics or by the morphology of most of the genital organs. In the laboratory, the two species were found to be reproductively incompatible and consequently may be considered sibling species. Differentiation of the two species can be accomplished only by careful dissection of the male and female genitalia and the demonstration of the presence of a vaginal pouch in *B. tenagophila* and its absence in *B. occidentalis*. There are, in addition, morphometric differences in the prepuce/penile sheath complex of the two species. Differentiation, therefore, is a highly complex process requiring a high level of skill in dissecting these organisms and rigid control of relaxation and fixation of the snails.

B. tenagophila is a recognized host of *Schistosoma mansoni*, whereas *B. occidentalis* appears to be a non-susceptible species (Paraense & Corrêa, 1982). The need for a method, less tedious than manual dissection, to differentiate the species is obvious.

A technique employing snail hemolymph and agarose gel electrophoresis (AGE) for the differentiation of the two species is described. The technique has been found to be simple, rapid, repeatable, cost effective and suitable for rural laboratories.

MATERIALS AND METHODS

Six strains of *B. tenagophila* and four strains of *B. occidentalis*, all from Brazil, were used in the study (Tab. 1). Maintenance of the snail populations was as previously described (Michelson, 1966).

Two methods were used for collecting hemolymph. When large amounts of hemolymph (50-100μl) were required and snails were expendable, hemolymph was collected from the pericardial cavity as described by Michelson (1966). If snails were required for breeding or for other studies, hemolymph was drawn by puncturing the mantle collar with the tip of a sharp forceps and collecting the pooled fluid with a fine-tipped micropipet. Approximately 10-50μl of hemolymph could be obtained with this method and snail mortality was generally less than 10%.

Fresh hemolymph was mixed in a proportion of 5:2 with a sample solution comprised of 80% glucose containing 5% glycerol and sufficient 0.5% bromphenol blue to give a dark purple color. Ten microliters of the mixture were used as the sample for agarose gel electrophoresis (AGE). AGE was conducted in either Mini-vertical or Mini-subhorizontal cells (Bio-Rad Laboratories) and both methods gave comparable results; however, the vertical system appeared to give sharper bands. In both systems, the gel matrix consisted of 0.8% agarose in 0.15 M tris-borate buffer, pH 8.5. Although we used an ultrapure DNA grade agarose (Bio-Rad Laboratories), pre-

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**Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799, U.S.A.

***Instituto Oswaldo Cruz, Departamento de Malacologia, Caixa Postal 926, 20001 Rio de Janeiro, RJ, Brasil.

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TABLE I

Species and the geographic origin of strains from which hemoglobin was obtained*

Species	Geographic Origin
Biomphalaria occidentalis	Barão de Melgaço, Mato Grosso
	Guaíra, Paraná
	Valparaíso, São Paulo
	Sena Madureira, Acre
Biomphalaria tenagophila	Joinville, Santa Catarina
	Rio de Janeiro, RJ
	Brasília, D.F.
	Taubaté, São Paulo
	São José dos Campos, São Paulo
	Vitória, Espírito Santo

*All snail populations were derived from those maintained by Dr. W.L. Paraense, Instituto Oswaldo Cruz, Rio de Janeiro.

liminary studies demonstrated that less chemically pure agaroses would suffice. Horizontal gels, 2mm in thickness, were prepared by pouring 15ml of melted agarose directly onto a Gel Bond(R) sheet (FMC BioProducts), 6.4cm x 11.0cm, containing a 10 comb well-spacer situated 1cm from a smaller edge. The gels were cured for 2hr at 5°C before use and AGE was run at 104V/70 min/25°C. Vertical gels, 1.5mm in thickness, were prepared in a casting cell and used approximately 9.3ml of agarose to make a gel 8cm wide x 7cm long. Gels were run at 104V/55 min/25°C. Routinely, ten sample wells were cast; however, the number may be varied to suit the needs of the investigator.

After electrophoresis was completed, gels were fixed in 12% acetic acid containing 5% glycerol for 1hr or until the bromphenol marker was no longer visible. Gels were then rinsed in deionized water and those run in a vertical cell were now mounted onto Gel Bond sheets. Permanent preparations were made by dehydrating the gels to a thin layer in accordance with the technique described by Saravia & Cook (1979). This consisted in covering the gels with a moist piece of filter paper, adding several layers of absorbent paper toweling, then placing an evenly distributed weight of 2kg on top of the gel-paper complex. After 30 min, the weight and the papers were carefully removed and the film further dried in a 37°C incubator for 4-6hr or held overnight at room temperature. Stain was not required as the red-colored hemoglobin bands were clearly visible. The preparations may be photographed or photocopied and the gel films can be stored indefinitely.

Migration distances were determined by noting the distance of the bands from the point of origin; however, Rf values could be determined also in relation to the bromphenol dye front.

RESULTS

Our results indicated that the hemoglobin portion of *B. occidentalis* hemolymph migrated faster than did that of *B. tenagophila*. In most runs the bands were separated by a distance of 1-2mm. Although some variation in absolute distance was noted between runs, the distance between the bands of the two species did not show appreciable variation. No differences were noted among the geographical strains of the same species. Variation appeared to be less in gels run in the vertical system than those done in the horizontal system.

In the 62 analyses done in the horizontal system, the hemoglobin band of *B. occidentalis* migrated at least 1mm farther than that of *B. tenagophila* in 55 comparisons (Fig. 1A). In five sample runs, the hemoglobin of the two species migrated equally or so closely to one another that they could not be differentiated. Only two sample runs were observed in which the *B. tenagophila* band migrated faster than its sibling species.

In all of the 42 analyses done in the vertical system, *B. occidentalis* hemoglobin migrated at least 1mm farther than did the hemoglobin of *B. tenagophila* (Fig. 1B). When the results from both systems were combined, it was found that *B. occidentalis* hemoglobin migrated faster than *B. tenagophila* 93.3% of the time, migrated at an equal rate with the latter species 4.8% of the time, and had a slower migration 1.9% of the time.

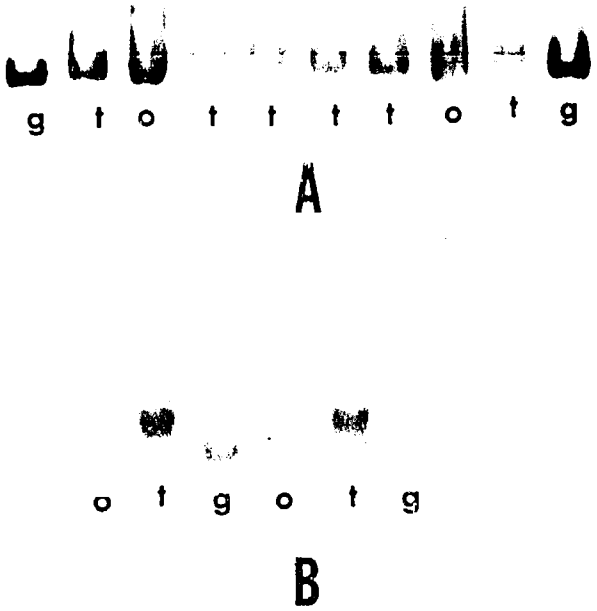

Fig. 1: Electrophoresis of snail hemolymph. A: Vertical gel; B: Horizontal gel; g: *Biomphalaria glabrata*; o: *B. occidentalis*; t: *B. tenagophila*. The vertical gel (A) was enhanced by staining with dianisidine and hydrogen peroxid due to faintness of the bands. The horizontal gel was unstained.

A limited number of experiments demonstrated that *B. glabrata* and *B. straminea*, both intermediate hosts of *S. mansoni* in Brazil, had hemoglobins which migrated faster than *B. occidentalis*; however, the two species could not be separated from one another in the present system.

DISCUSSION

Efforts to use hemolymph from species of planorbid snails as a taxonomic tool are not new and have been reviewed, in part, by Michelson (1973) and by Wright (1971, 1974). In past studies, electrophoretic analyses of hemolymph had been directed towards demonstrating differences in protein and isozyme patterns. Wright (1971) questioned the value of these techniques for taxonomic studies since he observed both quantitative and qualitative variations associated with age and/or size of individual snails. Subsequently, tissue extracts have replaced hemolymph as the sample of choice in applications of electrophoresis for taxonomic purposes.

The present study, however, demonstrates that the analyses of snail hemolymph hemoglobins by AGE may be a valuable adjunct to existing techniques for species identification. In *Biomphalaria* species, hemoglobin is a major constituent of the hemolymph and in some species may constitute as much as 40-65% of the total protein concentration (Michelson & Dubois, 1975). The hemoglobin fraction in these snails is characterized by being chromogenic, non-corpuscular, and of high molecular weight (1×10^6 or greater). Specific differences in hemoglobin mobility were not apparent in past studies, since the matrices employed for electrophoresis prevented or limited the migration of these molecules. AGE separates protein molecules by both charge densities and molecular seiving and, at concentrations of 0.6-0.8%, gels have pore sizes adequate for the migration of snail hemoglobins.

Our results indicate that differences in hemoglobin migration was a reliable criterion for separating the sibling species *B. occidentalis* and *B. tenagophila*, separation being demonstrated in 93.3% of the test samples. In addition, the mini-system has several advantages: (1) it permits the test snails to remain alive for use in other studies; (2) results are rapid; (3) the technique is simple and requires no special skills; (4) migration patterns can be demonstrated without the use of special stains or substrates; and (5) the method is relatively cheap, both in actual cost (estimated at $0.03-$0.08/sample) and materials required. Preliminary experiments suggest that the technique may be applicable to other groups of hemoglobin-bearing snails, and recent studies with African planorbids appear promising.

RESUMO

É descrito um método simples e rápido para distinguir as espécies crípticas *Biomphalaria tenagophila* e *B. occidentalis* por eletroforese em gel de agarose. A prova é feita com hemolinfa do molusco, permitindo a cor vermelha da fração hemoglobina visualizar os padrões de migração sem necessidade de recorrer a colorações específicas. Além disso, as amostras de hemolinfa podem ser obtidas sem sacrificar o molusco, que poderá ser usado para outros estudos ou para criação.

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