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SENSITIVITY OF DIAGNOSTIC TECHNIQUES IN DETERMINING THE PREVALENCE OF ANURAN TRYPANOSOMES

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ABSTRACT: Two hundred thirty-three leopard frogs (*Rana pipiens*) from Oshkosh, Wisconsin, USA, were divided into two groups and their blood examined for trypanosomes. In Group I (n = 157), where the blood was examined by the hematocrit centrifuge technique (HCT), 36 (23%) were infected with trypanosomes. Eighteen were infected with *Trypanosoma pipientis*, 13 with *Trypanosoma ranarum*, three with *Trypanosoma rotatorium* and two with mixed infections of *T. pipientis* and *T. ranarum*. In Group II (n = 76) the blood was cultured and also examined by HCT and wet mounts. Trypanosomes (*T. pipientis* and *T. ranarum*) were detected in 24 frogs (33%) using all three techniques. Eleven *T. pipientis* were detected by HCT, however none by culture and two by wet mounts. Twelve *T. ranarum* were detected by culture while only 10 were found by HCT and five by wet mounts. One *T. ranarum* infection detected by HCT was missed by culture because of bacterial contamination. The HCT was consistently better than wet mount examinations. It is suggested that the HCT be used whenever possible in future trypanosome surveys.

INTRODUCTION

There have been numerous reports of trypanosomes in anurans (see Reilly and Woo, 1982a). The prevalence varied from a low of about 2% (Fantham et al., 1942) to a high of 73% (Miyata et al., 1978). In the majority of studies, wet mounts and stained smears were used. This may in part explain the difference in prevalence reported.

No comparative study has been conducted to determine the relative sensitivity of the various parasitological techniques for detecting anuran trypanosomes. The purpose of the present study was to evaluate the relative sensitivity of three diagnostic techniques (in vitro culture, the hematocrit centrifuge technique, and wet mount technique) for the detection of amphibian trypanosomes. The leopard frog was chosen because at least three morphologically distinct species of trypanosomes have been recorded from this host (Diamond, 1965; Woo, 1969a; Werner and Walewski, 1976), and it is readily available in larger numbers.

MATERIALS AND METHODS

Leopard frogs were collected in the fall of 1980, from around Oshkosh, Wisconsin, USA. They were subsequently maintained in the laboratory for about 4 mo before the blood of 233 frogs was examined for trypanosomes using the following three techniques:

(1) In vitro culture: Frogs were anaesthetized in a 0.75% aqueous solution of tricaine methanesulfonate (MS222) (Syndel TMS, Syndel Laboratories Ltd., 8879 Selkirk Street, Vancouver, British Columbia V6P 4J6, Canada). Each frog was laid on its back and the ventral surface cleaned with 70% ethanol. A longitudinal slit was made to expose the heart. A 26 gauge needle attached to a sterile 1 ml tuberculin syringe was flamed and inserted into the ventricle. The syringe contained approximately 0.05 ml of sterile heparinized amphibian Ringer's solution (pH 7.2). As much blood as possible was slowly withdrawn from the heart. About 0.1 ml was aseptically dispensed into a modified Tobie's diphasic blood-agar medium which had been made with heat inactivated sterile bovine blood instead of rabbit blood (Tobie et al., 1950). Seven milliliters of blood agar was dispensed into a disposable 250 ml Falcon flask (Fisher Scientific Co., 184 Railside Road, Don Mills, Ontario M3A 1A9, Canada), slanted and allowed to solidify at room temperature. Approximately 8 ml of amphibian Ringer's solution (pH 7.2) was added aseptically after solidification. The cultures were kept at room temperature (about 20 C) for at least 5 wk. They were examined weekly using an inverted microscope.

(2) The haematocrit centrifuge technique: Three tubes of blood in heparinized capillary tubes (Fisher Scientific Co., 184 Railside Road, Don Mills, Ontario M3A 1A9, Canada) were centrifuged at 11,500 rpm for 4 min. After centrifugation, the tubes were examined with a compound microscope (objective 10×, eye piece 10×) for trypanosomes (Woo, 1969b). The tubes with trypanosomes were cut at the junction of the buffy coat and plasma. Smears were made from the buffy coat and air dried. These were fixed in absolute methanol for 5 min, followed by a further 5 min in 10% buffered formalin (Lehmann, 1964). The smears were stained in Giemsa's stain for 45 min.

(3) Wet mount examination: A drop of fresh blood (about 0.03 ml) was dispensed onto a microscopic slide and covered with a cover-slip. At least 80 fields (objective 10×, eye piece 10×) were examined.

The frogs were randomly divided into two groups. In Group I, the blood of 157 frogs was examined only by the hematocrit centrifuge technique. In Group II (76 frogs), the blood was (i) cultured aseptically and examined by (ii) the hematocrit centrifuge technique, and (iii) wet mount.

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RESULTS

Group I: Thirty-six frogs (23%) were infected with three morphologically distinct trypanosomes. Eighteen were infected with *Trypanosoma pipientis* Diamond, 1950, 13 with *Trypanosoma ranarum* (Lankester, 1871) Danilewsky, 1885, three with *Trypanosoma rotatorium* (Mayer, 1843) Laveran and Mesnil, 1901, and two with a mixed infection of *T. pipientis* and *T. ranarum*. The detailed descriptions of the three trypanosomes are given elsewhere (Diamond, 1965; Woo, 1969a). Representative slides of each of the three species were deposited in the National Museum of Natural Science, Ottawa, Ontario K1A 0M8, Canada. The specimen numbers are NMCIC(P) 1982–1612 for *T. pipientis*, NMCIC(P) 1982–1613 for *T. ranarum*, and NMCIC(P) 1982–1614 for *T. rotatorium*.

Group II: Trypanosomes (*T. pipientis* and *T. ranarum*) were detected in 24 of the 76 frogs (33%) using all three techniques (Table 1). None of the 11 *T. pipientis* infections (detected by the hematocrit centrifuge technique) became established in culture. However, 12 *T. ranarum* infections were detected by the culture technique compared to only nine by the hematocrit centrifuge technique. Ten of the 76 culture bottles (with medium) inoculated with anuran blood were contaminated. Two of these had trypanosomes, one with *T. pipientis* and the other with *T. ranarum*. The hematocrit centrifuge technique detected 21 infections, compared with only seven by wet mount examinations.

TABLE 1. The sensitivity of diagnostic techniques for the detection of anuran trypanosomes.

Trypanosome species	All three combined techniques	Culture technique only	Wet mount examination only	Hematocrit centrifuge only
T. pipientis	11[a]	0	2	11
T. ranarum	13	12[b]	5	10[c]
Total	24	12	7	21

[a] Number of infections detected.

[b] One infection was missed as a result of bacterial contamination; this was detected by the hematocrit centrifuge technique.

[c] Three infections were missed but these were detected by the culture technique.

DISCUSSION

The present study confirms earlier studies (Diamond, 1965; Woo, 1969a; Werner and Walewski, 1976) that leopard frogs are infected with *T. pipientis*, *T. ranarum* and *T. rotatorium*. Diamond (1965) recorded *T. pipientis* in 27% of 74 leopard frogs and 34% of 129 *R. pipiens* tadpoles in Michigan. However, Werner and Walewski (1976) found *T. pipientis* in only 8% of 24 leopard frogs using wet mount and thin smear examinations. Woo (1969a), using the hematocrit centrifuge technique, detected *T. pipientis* in 13% of 151 *R. pipiens* examined in southern Ontario. In the present study 12% of 239 leopard frogs were infected with *T. pipientis*. Werner and Walewski (1976) detected *T. ranarum* in 17% of 24 leopard frogs while Woo (1969a) found it in 3% of 151 frogs in southern Ontario. *T. rotatorium* was recorded from 71% of 24 and 5% of 151 leopard frogs in earlier surveys (Woo, 1969a; Werner and Walewski, 1976). The prevalence of *T. rotatorium* was considerably lower in the present study. These differences may also reflect differences in prevalence of the trypanosomes in different populations of frogs.

The hematocrit centrifuge technique (Bennett, 1962) modified by Woo (1969b) was more sensitive than wet mount examinations for the detection of anuran trypanosomes. This confirms an earlier study when the technique was first used on human trypanosomiasis (Woo, 1970). The technique has since been used successfully for the parasitological diagnosis of African human trypanosomiasis (Onyango and Mbwabi, 1970; Woo, 1970, 1971; Felgner et al., 1981) and animal trypanosomiasis (Woo and Kauffmann, 1971; Rukmana, 1972; Leeflang et al., 1978). Although the technique is sensitive, its sensitivity varied with trypanosome species (Woo and Rogers, 1974). This is confirmed in the present study as three *T. ranarum* infections (detected by culture) were missed by this technique.

Trypanosoma pipientis did not multiply in the diphasic blood-agar medium used. Although this medium was initially devised for the culture of the African trypanosomes (Tobie et al., 1950), it has since been modified to culture other mammalian trypanosomes (Woo et al., 1970; Bower and Woo, 1982), an avian trypanosome (Woo and Bartlett, 1982), a reptilian trypanosome (Woo, 1969c) and anuran trypanosomes (Reilly and Woo, 1982b). In the present study, *T. ranarum* multiplied readily in the medium but *T. pipientis* would not. Diamond (1965) also found that *T. pipientis* would not establish

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in several isotonic media, but would in a hypotonic medium. The culture technique, though more sensitive than the hematocrit centrifuge technique in detecting *T. ranarum*, has several obvious limitations. Not all trypanosomes would multiply readily in a single medium and blood has to be collected and inoculated aseptically into a suitable culture medium. In the present study, 10 of 76 cultures were contaminated. Trypanosomes do not survive and multiply in contaminated cultures; consequently the culture technique is not practical for most surveys.

The hematocrit centrifuge technique is more sensitive and rapid than the examinations of wet mounts and stained blood smears. This technique should be used whenever possible in future surveys of anuran trypanosomes.

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