Possible association between *Taenia solium* cysticercosis and cancer: increased frequency of DNA damage in peripheral lymphocytes from neurocysticercosis patients

Luis A. Herrera[1], Tzutzuy Ramirez[1], Ulises Rodríguez[2], Tere Corona[2], Julio Sotelo[2], Marcelino Lorenzo[3], Francisco Ramos[3], Irmgard Verdorfer[4], Erich Gebhart[4] and Patricia Ostrosky-Wegman[1] *[1]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), México, D.F., Mexico; [2]Instituto Nacional de Neurología y Neurocirugía, Secretaría de Salud, México, D.F., Mexico; [3]Unidad de Neurología, Hospital General de México, México, D.F., Mexico; [4]Institut für Humangenetik der Universität Erlangen-Nürnberg, Erlangen, Germany*

Abstract

Helminths, particularly some *Schistosoma* species, have been associated with cancer in humans. Neurocysticercosis, produced by cysticerci of the helminth *Taenia solium*, has been associated with the emergence of brain tumours and haematological malignancies. Local tumours, such as glioblastoma, could be explained by the induction of DNA damage in cells surrounding the cysticercus and chronically exposed to an inflammatory host response. However, systemic effects such as haematological malignancies are not easy to understand. The present work was conducted in Mexico to find out whether DNA damage arises in peripheral lymphocytes in patients with neurocysticercosis. We utilized a highly sensitive technique to analyse chromosomal aberrations, in-situ hybridization with probes against chromosomes 1, 2 and 4, and in addition the blocked-cytokinesis technique was used to determine the formation of micronuclei, a peculiar form of DNA damage. The study was made in lymphocytes from 8 patients before and after the administration of praziquantel, 1 of the 2 drugs used for neurocysticercosis treatment. The frequencies of chromosome aberrations and micronuclei in peripheral blood lymphocytes were higher in the infected patients as compared to those observed both in healthy donors and in the group of patients after praziquantel therapy. Our results suggest that chromosome aberrations induced in peripheral cells during neurocysticercosis could be associated with the development of haematological neoplasias.

Keywords: neurocysticercosis, *Taenia solium*, cancer, chromosome aberrations, Mexico

Introduction

Several infectious agents such as viruses, bacteria, and helminths have been implicated in the development of human cancer (IARC, 1994). Among helminths, *Schistosoma haematobium*, *S. japonicum*, and *Opisthorchis viverrini* are associated with malignant neoplasms in urinary bladder, liver, and bile ducts (IARC, 1994). Neurocysticercosis (NCC), a frequent parasitic disease of the nervous system, is caused by cysticerci of the helminth *Taenia solium*; NCC has recently been associated with local malignant tumours, particularly glioblastoma multiforme (DEL BRUTTO *et al.*, 1997).

Studies to determine the possible mechanisms leading to carcinogenesis by *Schistosoma* have demonstrated a high frequency of DNA damage in urothelial cells near the infection site (ANWAR & ROSIN, 1993; ROSIN *et al.*, 1994). The local chronic inflammatory response, a feature frequently observed in helminthiasis, has been postulated as a mechanism to explain carcinogenesis by parasites (ISHII *et al.*, 1994; OHSHIMA & BARTSCH, 1994), by inducing either direct DNA damage, an altered metabolism to xenobiotics or an impaired proliferation of cells surrounding the parasite (GENTILE & GENTILE, 1994).

Our studies with NCC, however, suggest that, apart from the local effects produced by parasites, some systemic alterations also due to cysticerci could be found in cells from infected individuals. In fact, an epidemiological study has associated NCC with the emergence of neoplasias outside the nervous system, e.g., malignant haematological diseases (HERRERA *et al.*, 1999). In that study, 1271 autopsy files of a general hospital in Mexico City were reviewed. Files were separated into 2 groups: the cases group which included patients whose autopsies revealed any malignant neoplasia, and a control group consisting of all patients with no histopathological indication of cancer. NCC was more frequent in cases with malignant haematological diseases than in controls. The odds ratio for this association was 3·5 with 95% confidence interval 1·2–9·8.

In the present work we determined whether active NCC (those cases where the cysticercus is alive) is directly involved in the production of chromosomal damage in peripheral lymphocytes. We measured the frequency of chromosome aberrations in peripheral lymphocytes from patients with active NCC before and after the administration of praziquantel (PZQ). In addition, we determined the lymphocyte proliferation kinetics (LPKs) since there are some indications that helminthiasis may also cause impairment of cell proliferation in the host (GENTILE & GENTILE, 1994).

In addition, since the genotoxicity of PZQ itself has been questioned and not totally discarded (for review see MONTERO & OSTROSKY, 1997), we evaluated the effects of PZQ on the frequency of micronuclei and on cell proliferation when added to cultured human lymphocytes at doses similar to plasma levels of PZQ observed in patients during treatment.

Material and Methods

In-vivo study

The study included 8 patients with NCC, 5 women and 3 men. The protocol was approved by the Ethics Committee of the Instituto Nacional de Neurología y Neurocirugía in Mexico. All individuals were informed about the objectives of the present study and written consent was obtained before their incorporation in the trial. Median age was 37·5 years (range: 20–78). Only those patients without previous cestocidal treatment were selected. The treatment regimen with PZQ was decided according to the localization of cysticerci: 75 mg/kg in a single-day therapeutic schedule (CORONA *et al.*, 1996) for those patients presenting cysticerci within the brain parenchyma, and 50 mg/kg daily for 15 days for those patients with subarachnoid cysticerci (SOTELO & JUNG, 1998). Blood samples were obtained by venepuncture before and 1 week after PZQ therapy.

Blood samples from 11 uninfected healthy individuals, 5 women and 6 men, were used as controls. Median age was 29 years (range: 20–48). These individuals were asymptomatic and a computed tomography of the brain

Address for correspondence: Luis A. Herrera, Instituto de Investigaciones Biomédicas, Departamento de Genética y Toxicología Ambiental, UNAM, P. O. Box 70-228, Ciudad Universitaria, 04510, México D.F., Mexico; phone +525 622 38 46, fax +525 622 33 65, e-mail metil@hotmail.com

was used to discard the presence of any silent infection with cysticerci.

Chromosome aberrations were analysed using the chromosome in-situ suppression hybridization technique (CISS). CISS is an easy technique which can detect chromosome aberrations by allowing visualization of colour changes as shown in Figure 1 (LUCAS *et al.*, 1989; CREMER *et al.*, 1990); several laboratories have demonstrated that chromosome painting is a valid method of quantifying chromosome aberrations (NATARAJAN *et al.*, 1992; TUCKER *et al.*, 1993, 1995; ELLARD *et al.*, 1995). Whole blood (1 mL) was cultured in 9 mL of RPMI culture medium supplemented with 10% fetal calf serum, and stimulated with phytohaemagglutinin (0·4 mL) during 48 h at 37°C. After 20 min of an 0·075 M KCl hypotonic treatment, cells were fixed with methanol:acetic acid (3:1), and chromosome preparations were made by the standard air-drying procedure. The slides were kept in 70% ethanol at −20°C before further use for in-situ hybridization. Specific labelling of the 3 selected chromosomes was performed with a cocktail containing DNA probes for chromosomes 1 (red), 2 (green) and 4 (yellow) as shown in Figure 1. Chromosome aberrations were analysed by a fluorescence microscope, determining the frequency of chromosome and chromatid breaks, as well as balanced and unbalanced translocations in at least 500 mitoses per individual (NEUBAUER *et al.*, 1996).

For the micronuclei assay, 0·5 mL of whole blood was cultured in 6 mL RPMI medium and stimulated with phytohaemagglutinin (0·2 mL) during 72 h. Cytochalasin-B (3 μg/mL) was added 28 h before harvesting. Cells were gently fixed with methanol:acetic acid (3:1) and dropped on to clean slides (FENECH & MORLEY, 1985). Slides were stained with methylene blue plus eosin (Wright's colorant), and the frequency of micronuclei was estimated in at least 1000 binucleated cells per donor.

The LPKs were measured in whole blood lymphocytes (0·5 mL) cultured in 6 mL RPMI containing bromodeoxyuridine (32 μM), and stimulated with phytohaemagglutinin during 72 h. Demecolcine was added to the cultures for the last 2 h and, after 30 min of a hypotonic treatment (KCl 0·075 M), cells were fixed with methanol:acetic acid (3:1). The cells were dropped on to slides and stained by the fluorescence plus Giemsa technique to differentiate sister chromatids (PERRY & WOLFF, 1974). The LPKs were determined in 100 consecutive metaphases as the frequency of cells which performed 1 (M1 cells), 2 or 3 and more mitotic divisions.

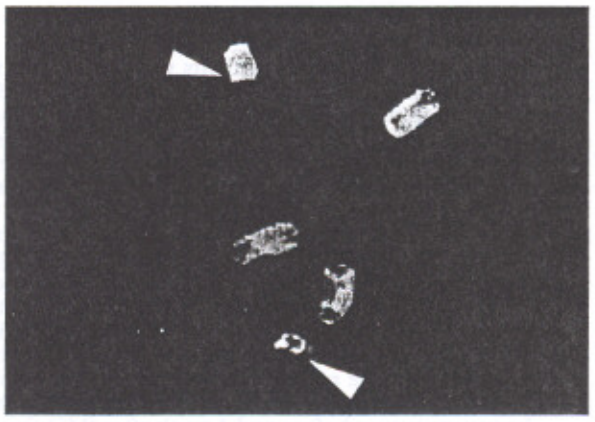

Fig. 1. Three-colour (red, chromosome 1; green, chromosome 2; yellow, chromosome 4) chromosome in-situ suppression hybridization of a lymphocyte metaphase showing a balanced translocation (arrows), which accounts for 2 breaks.

In-vitro analysis

In 3 independent experiments whole blood samples from 1 healthy donor were used to determine the frequency of micronuclei induced *in vitro* by PZQ. The PZQ doses utilized were within the range found in the plasma from patients with NCC: 10^{-7}–10^{-6} M. Blood (0·5 mL) was cultured in 6 mL RPMI and stimulated with 0·2 mL phytohaemagglutinin during 72 h. Twenty-eight hours before harvesting, cytochalasin-B was added (3 μL/mL), and 4 h later cells were treated with PZQ dissolved in dimethylsulphoxide (0·001 v/v final solvent concentration). Cells were gently fixed with chilled methanol:acetic acid (3:1) and dropped on to slides. After 24 h, slides were stained with Wright's colorant. One thousand binucleated cells were analysed for the presence of micronuclei.

The LPKs were measured in whole blood (0·5 mL) cultured in 6 mL RPMI containing bromodeoxyuridine (32 μM), and stimulated with phytohaemagglutinin during 72 h. Treatment with PZQ was carried out during the last 24 h of culture. Harvesting and staining procedures were carried out as mentioned above. LPKs were determined in 100 metaphases as the frequency of cells which performed 1 (M1 cells), 2 or 3 and more mitotic divisions.

Statistical analysis

The frequencies of chromosomal aberrations, micronuclei and M1 cells of the infected individuals, both before and after treatment with PZQ, were compared to those of the control group with a Mann–Whitney test. The Wilcoxon test for paired samples was utilized to compare results observed in the group of patients with NCC before and after treatment with PZQ. Two-tailed P values of < 0.05 were considered significant. An ANOVA was used for results from the study *in vitro*. P values < 0.05 were considered as significant.

Results

In-vivo study

Total frequency of breaks (Fig. 2a) represents the sum of all breaking events observed as chromosome and chromatid breaks (1 break per aberration), or balanced and unbalanced translocations (2 breaks per aberration). A higher frequency of breaks was observed in NCC patients before PZQ treatment, median 8·75, range 3–17·5, when compared to the frequency obtained in healthy volunteers, median 2, range 0–5·2 ($P = 0.02$). Although after the administration of PZQ the total frequency of breaks in cells from NCC patients was higher, median 3·8, range 0–9, than in cells from control individuals, the difference was not significant ($P = 0.4$).

The quantification of binucleated cells containing micronuclei (Fig. 2b) revealed a significant difference between the frequency of damage detected in the group of patients before treatment, median 46, range 18–100, and in healthy donors, median 20, range 15–24 ($P = 0.01$). After treatment with PZQ, the occurrence of micronucleated cells returned to normal values, median 20·5, range 13–28; therefore, it was significantly different from that in patients before PZQ therapy ($P = 0.03$). A similar circumstance was observed in the analysis of LPKs (Fig. 2c). The percentage of M1 cells after 72 h of culture was higher in samples from patients prior to PZQ administration than the percentage obtained both in healthy donors ($P = 0.008$) and in patients after PZQ administration ($P = 0.04$).

In-vitro analysis

The mean frequency of micronucleated cells of 3 independent experiments using different concentrations of PZQ is shown in Figure 3a. Although there is an increase in the frequency of binucleated cells with micronuclei, at the higher concentration, the difference was statistically not significant, even at doses as high as 10^{-4} M ($P = 0.65$). With respect to LPKs, no change was

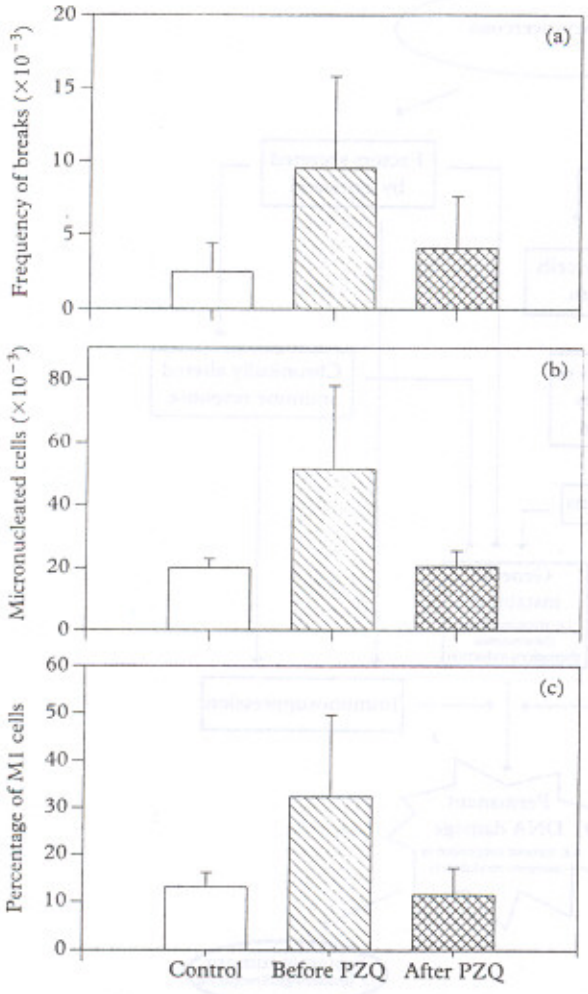

Fig. 2. Total frequency of breaks (a), micronucleated (b) and M1 cells (c) in lymphocytes from patients with neurocysticercosis before and after the administration of praziquantel (PZQ). The control group includes healthy volunteers who were not treated with PZQ. See the text for details of the treatment and methodology. The bars represent SD.

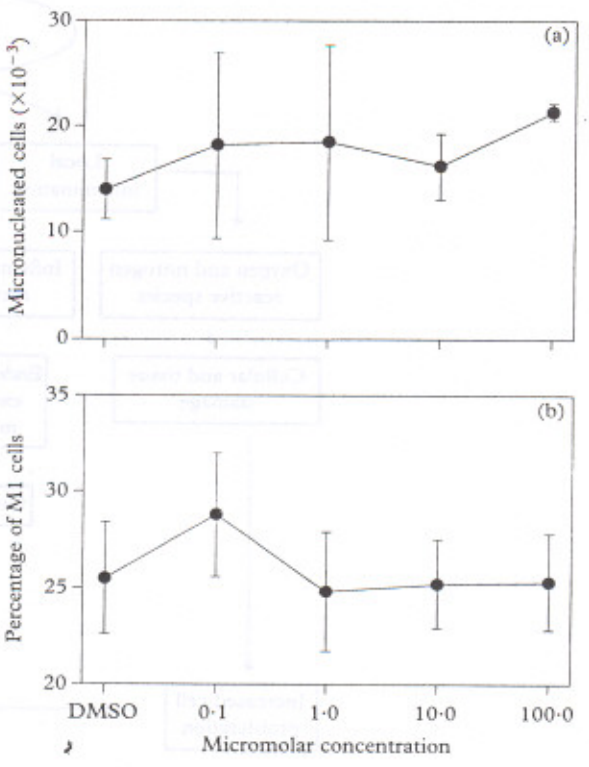

Fig. 3. Frequency of micronucleated (a) and M1 (b) cells from lymphocyte cultures treated with praziquantel. DMSO, dimethylsulphoxide. See the text for details of the methodology. The bars represent SD.

observed in the frequencies of M1 cells at different concentrations of PZQ tested ($P = 0{\cdot}39$; Fig. 3b).

Discussion

Neurocysticercosis is the most frequent parasitic disease to affect the central nervous system. It is endemic in many parts of the world, particularly in Latin America, Africa, and Asia (FLISSER, 1994). It is also frequent in developed countries with high rates of immigration from endemic areas. NCC, as well as other helminthiasis, has been associated with the development of neoplasms in tissues surrounding the parasite (DEL BRUTTO *et al.*, 1997), and with the generation of malignancies outside the central nervous system (HERRERA *et al.*, 1999). The latter was an event anticipated by us in a previous study since lymphocytes from pigs infected with *T. solium* cysticerci presented a higher frequency of DNA damage as compared to lymphocytes from uninfected animals (HERRERA *et al.*, 1994). In the present study, we analysed the induction of chromosomal damage in peripheral lymphocytes of patients with NCC before and after the administration of PZQ, 1 of the 2 drugs indicated in the chemotherapy against NCC. To discard any genotoxic effect by PZQ itself, we determined the frequency of genetic damage induced by PZQ in cultured lymphocytes from a healthy donor at concentrations that resemble PZQ plasma levels in patients with NCC (10^{-6}–10^{-7} M; SOTELO & JUNG, 1998).

Results indicated that the rate of cells with DNA damage observed in patients with NCC, revealed either as micronuclei or chromosome aberrations, did not increase after the administration of PZQ, regardless of the therapeutic scheme. Moreover, both frequencies of damage were lower among patients after the treatment than before. Similarly, the response of lymphocytes to mitogen stimulation was slower in the cells from patients before treatment. The frequency of genotoxic damage found in the group of patients before therapy was significantly higher than that observed in healthy donors. The frequencies of micronuclei and chromosome aberrations in infected patients without treatment were significantly higher than those obtained in healthy volunteers.

The effects of addition of PZQ to cultures of lymphocytes from a healthy donor also suggest that PZQ lacks genotoxicity, and PZQ did not modify the LPK. We have reported that PZQ has no effect on the rate of hypoxanthine phosphoribosyl transferase mutations, and sister chromatid exchanges in mammalian cells, although it induced an increase in the proportion of cells with single-strand DNA damage (HERRERA *et al.*, 1998). These results suggest that PZQ is not genotoxic at the usual therapeutic doses, and point out the necessity of exploration on the potential mechanisms of parasites as inducers of genotoxic damage.

Some biological conditions frequently present during cysticercosis that could be responsible for the observed DNA damage and which could increase the risk for malignant neoplasms are shown in Figure 4. The inflammatory response present in chronic infections has been previously analysed as a possible cancer risk factor (OHSHIMA & BARTSCH, 1994). Chronic inflammation is present in most NCC patients (ESCOBAR, 1983);

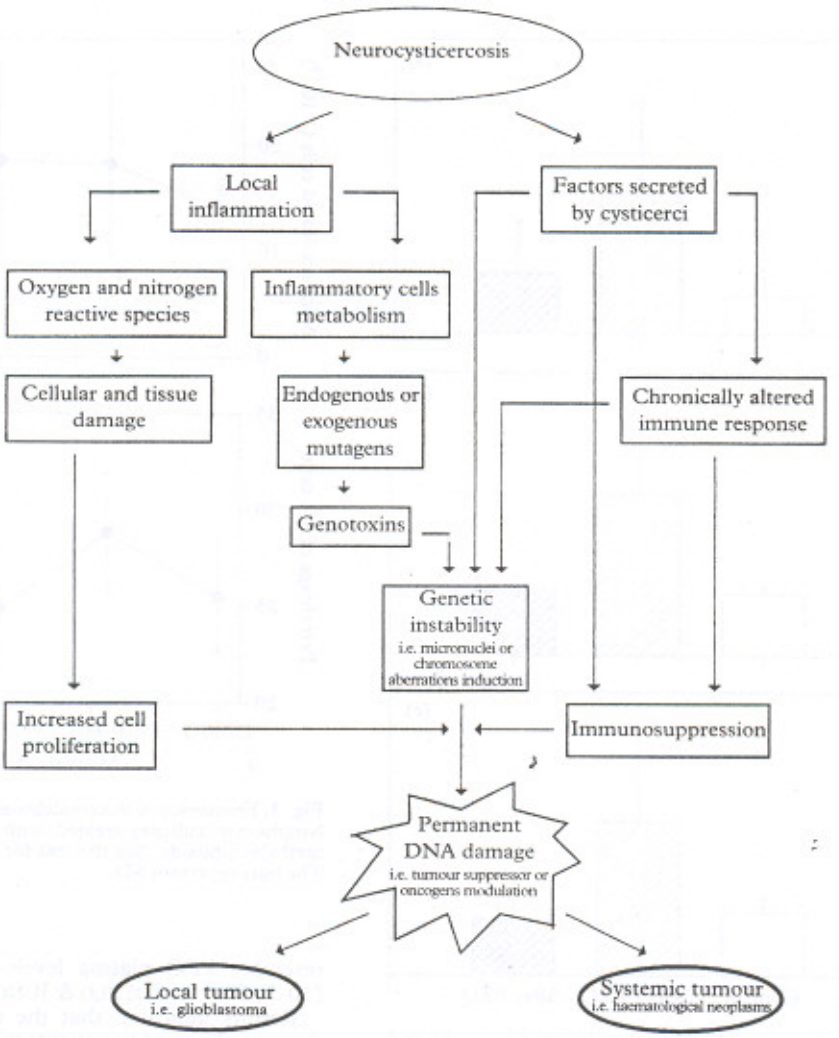

Fig. 4. Possible mechanisms by which neurocysticercosis could induce DNA damage and cancer development both locally and systemically.

inflammatory cells produce reactive oxygen and nitrogen species capable of causing significant damage to normal cells in tissues surrounding the site of parasitic infection, and this event could lead to genetic instability (OHSHIMA & BARTSCH, 1994). However, local accumulation of inflammatory cells could assimilate nearby mutagens, metabolize them, and release genotoxic agents that can cause damage in surrounding tissues (GENTILE & GENTILE, 1994). A cell proliferative process induced to repair the tissue and the cellular damage produced by the inflammatory response may result in an increased frequency of mutations (ROSIN *et al.*, 1994); some of these mutations could then lead either to oncogene activation or to the inactivation of suppressor genes thus increasing the risk for tumour formation.

Local inflammation might not be the only mechanism by which cysticerci could increase the risk for cancer development. NCC has also been associated with alterations in the immune response. In fact, it has been reported that *Taenia* cysticerci secrete several factors that can interact with host cells impairing the immune response (for a review see WHITE *et al.*, 1992). When these alterations are chronic, as is the case with NCC patients, they could ultimately generate either a systemic immunosuppression or a set of immune cells with genetic instability and chromosome aberrations. Epidemiological studies have found an indicative association between chromosome instability in lymphocytes and the risk for lymphatic and haematopoietic malignancies (BONASSI *et al.*, 1995; HAGMAR *et al.*, 1998). Our results have shown that NCC patients present a higher frequency of chromosome damage in peripheral lymphocytes before treatment, suggesting an unstable status of their DNA which may cause a future development of diseases with a genetic component such as cancer.

Moreover, results from previous studies indicated that a factor isolated from in-vitro secretions of *T. solium* cysticerci cause morphological transformation of SHE cells (HERRERA *et al.*, 1994); recently we have found that this small RNA molecule induces micronuclei in human cultured lymphocytes at doses similar to, or even lower than, those able to transform SHE cells, indicating that DNA damage in host cells could be directly induced by molecules secreted by cysticerci.

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Correction

G. Snounou *et al.* Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Transactions*, **93**, 369–374. In Table 1, the primers 'M1-KF' and 'M1-KR' should both be referred to as 'K1 family-specific - Nest 2' in the 'Notes' column, whereas the primers 'M1-MF' and 'M1-MR' should both be referred to as 'MAD20 family-specific - Nest 2' in the 'Notes' column.

All the amplification reactions performed to amplify polymorphic regions of *msp1*, *msp2* and *glurp*, were performed in the presence of 1 mM $MgCl_2$.