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TOXOPLASMA GONDII: A RAPID METHOD FOR THE ISOLATION OF PURE TACHYZOITES. PRELIMINARY CHARACTERIZATION OF ITS GENOME

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A rapid and simple technique for the purification of Toxoplasma gondii *tachyzoites was developed. Highly purified parasites were obtained from the peritoneal exudates of infected mice by means of two consecutive discontinous sucrose gradients run at low speed (10,000xg, 30 min). Parasites obtained by this method conserved its biological activity. Hybridization studies with DNA from healthy mice and from purified tachyzoites preparations demonstrated that* Toxoplasma gondii *tachyzoites DNA could be obtained with better than 90% purity. Preliminary studies with DNA endonucleases showed the presence in the tachyzoites genome of highly repetitives sequences.*

Key words: *Toxoplasma gondii* – tachyzoites purification – DNA repetitive sequences

Toxoplasma gondii, an obligated intracellular protozoan parasite, is the aethiologic agent of toxoplasmosis, a worldwide distributed disease that affects about one third of the world population as well as domestic animals. Most infected humans remain asymptomatic, however in immunocompromised individuals and in neonates severe consequences ranging from neurological sequelae or severe faetal damage in the congenital form, to lymphadenopathies or meningoencephalitis may result (McLead & Remington, 1980).

The life cicle of *T. gondii* consists of both sexual and asexual forms. The asexual life cicle involving extracellular and intracellular forms (which differ in several biochemical and immunological aspects), take place in intermediate hosts, like humans. The sexual life cicle which results in the shedding of millions of oocyst can only take place in animals of the feline family (i.e. cats). It is possible to obtain pure parasites by collecting oocyst from cats faeces but the procedure is cumbersome, the cats are expensive to breed and yields are very low (Dubey et al., 1970). Therefore most research on *T. gondii* is carried out using the tachyzoites form (asexual form) which can be harvested in large quantities from the peritoneal cavity of experimentally infected mice.

However, biochemical studies on *T. gondii* have been hampered by the difficulty in obtaining host cell free parasite preparations. Murine cells contaminations of parasites samples is a serious handicap for the isolation of pure *T. gondii* DNA. The fact that the genome of higher eukaryotes is about 100 fold larger than the protozoan one implies that 1% host cell contamination would yield up to 50% contaminated DNA preparation (McCutchan et al., 1984).

Using CsCl/Hoechst 33258 gradients, Johnson et al. (1986) achieved a tachyzoite DNA purification of about 90%. However, up to date, no attempts have been made to purify tachyzoites to approach biochemical, immunological and molecular studies of the parasites.

In this paper we report a very simple technique for the isolation of highly purified *T. gondii* tachyzoites and a partial characterization of the parasite genome is, in addition described herein.

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MATERIALS AND METHODS

Chemicals and enzymes – All chemicals were molecular biology grade and were purchased from either Bethesda Research Laboratories (BRL), Bethesda, Maryland; Carlo Erba, Italy; or Sigma Chemical Co., USA. DNA endonuclease were all from BRL.

Parasites – *Toxoplasma gondii* tachyzoites of RH strain were grown in the peritoneal cavity of Balb/c mice. Peritoneal fluids were extracted three days after the inoculation and collected in cold PBS (20 mM Phosphate, 150 mM NaCl, pH 7.2) plus 1 mM $CaCl_2$. In all cases pellets containing 10^9-10^{10} tachyzoites were obtained.

Parasite purification – Sucrose step gradients were prepared by carefully layering equal volumes of 40-50-60% sucrose in PBS-Ca solution in either 40 or 4 ml centrifuge tubes. The parasite suspension (2 ml) were loaded on top of the gradient and centrifuged at 10,000xg for 30 min at 4 °C. Thereafter, gradients were fractionated into 0.5 ml aliquots and washed three times in the same PBS-Ca buffer. Washings were performed by centrifugation (3,000xg, 10 min). Fractions containing enriched parasite suspensions were pooled and centrifugated in 4 ml tubes under the conditions described above. All parasite preparations were finally washed once and stored at – 70 °C.

Parasite DNA extraction – Parasites were lysed in TSE buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM ClNa, pH 7.2) plus 1% Sodium Dodecyl Sulphate (SDS) and incubated with 500 µg/ml of predigested pronase at 37 °C for 1h. The DNA was successively extracted with equal volumes of phenol, phenol/chloroform (1/1), chloroform, and ether, and then precipitated with sodium acetate-ethanol at – 20 °C for 16h and collected by centrifugation (10,000xg, 30 min). After centrifugation the DNA pellet was washed once with ethanol 70% and resuspended in 200 µl of 10 mM Tris-HCl pH 7.2; 0.1 mM EDTA. RNAase A (20 µg/ml) was added and the DNA solution was incubated for 1h at 37 °C and then precipitated. Aproximately 100 µg of DNA were obtained from each sample.

Dot blot analysis – DNA from each band of the sucrose gradient and DNA from *Escherichia coli*, uninfected mouse and impure *T. gondii* preparation or whole cells, were spoted onto nitrocellulose filters. Denaturation and neutralization were performed on Whatman 3 mm paper soaked with 0.5 M NaOH and Tris-HCl pH 7.2 respectively, followed by two treatments with 1.5 M NaCl and Tris-HCl pH 7.2. Filters were then washed in 3x SSC (1X SSC: 0.15 M ClNa; 0.15 M Na Citrate) and dried for 3h at 80 °C. Prehybridization (2h) and hybridization (18h) were performed in 6x SSC 0.25% plus low fat milk at 65 °C. The probes (DNA extracted from parasites of the middle band of the second gradient and a cloned highly repetitive DNA sequence from mouse genome) were labeled by Nick Translation (Maniatis et al., 1982).

Southern blot analysis – 3 µg of mouse and *T. gondii* DNA were digested with restriction enzymes according with manufacturer's recommendation. The digest were electrophoresed in 1% agarose gels (1V/cm for 18h), stained with ethidium bromide, blotted onto nitrocellulose (Maniatis et al., 1982) and hybridized with mouse and *T. gondii* genomic DNA probe labeled with 32p dATP under the same conditions described above.

RESULTS

Peritoneal exudates of mice infected with *T. gondii* showed a large contamination with host cellular material, hence, it was subjeted to 40-50-60% sucrose step gradient centrifugation and three clearly separated bands were observed (Fig. 1A). The contaminating mouse cells were detected by hybridizing the different fractions with a mouse genomic 32p probe. Such an analysis from the first centrifugation showed a major radioactive peak at the region corresponding to the bottom band and a minor one at the region of the middle band (Fig. 2A, dotted arrow). Optical microscope observation showed that tachyzoites appeared concentrated in the middle band (Fig. 1A, band 2), whereas the upper band (Fig. 1A, band 3) contained which appeared as cellular debris. However it is clear that parasite preparation obtained from band 2 still carried detectable quantities of mouse cells contaminants. A parasite preparation with no detectable mouse cells contamination was achieved by submitting material from band 2 to a second sucrose gradient centrifugation (Fig. 1B). No detectable hybridization signal with the mouse genomic 32p DNA probe was obtained at the region of the band 2 of the

second gradient (Fig. 2B), but when DNA extracted from band 2 of the second gradient was isolated, labeled and used as a probe, radioactivity was found associated at the homologous region (Fig. 2C), and no detectable signal was found in the region of the band 1. This is an additional indication that material of band 2 of the second gradient was not contaminated with mouse material.

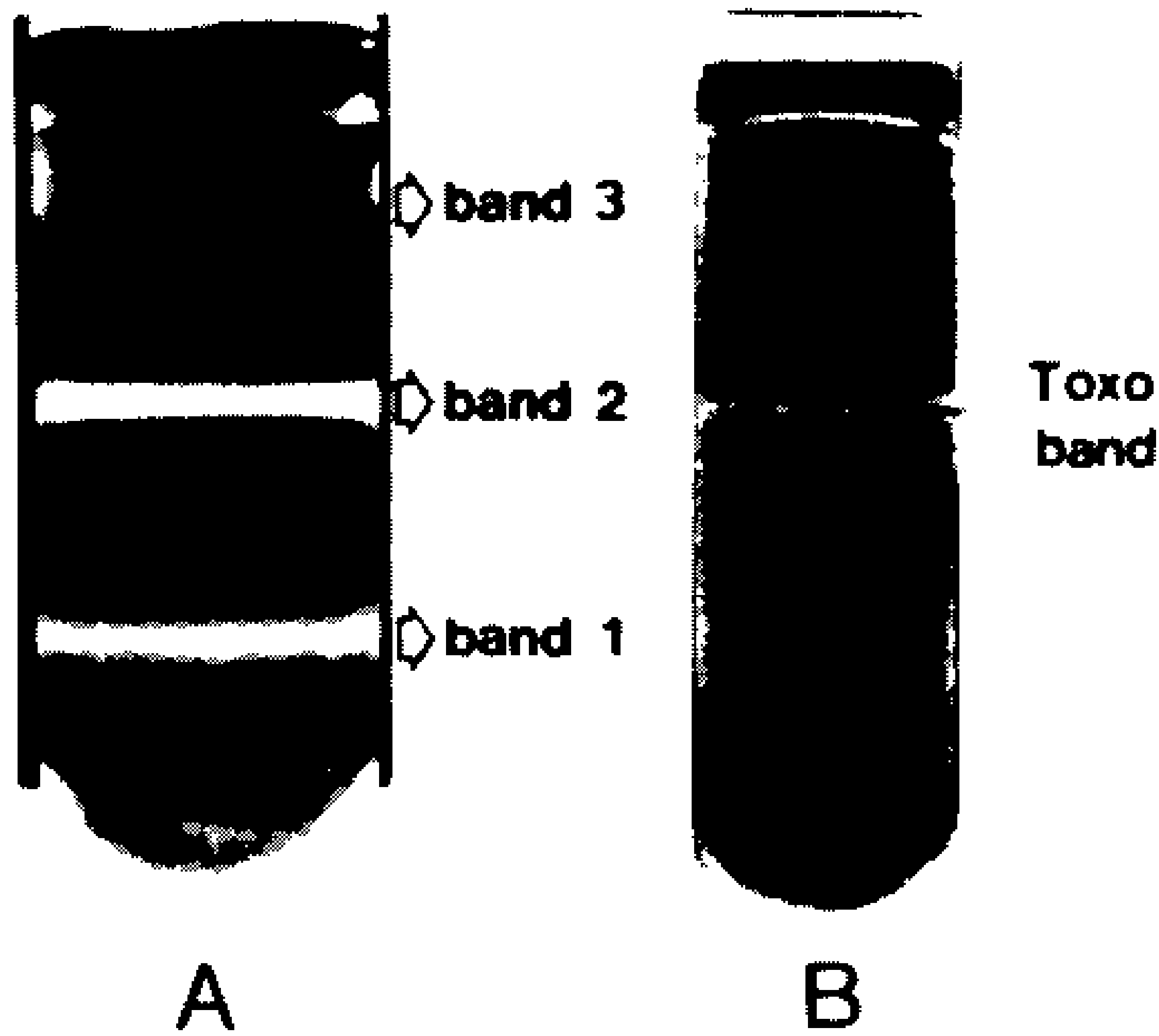

Fig. 1: sucrose step gradient of the peritoneal exudate from mice infected with *Toxoplasma gondii* tachyzoites. A – Peritoneal exudates obtained from five experimental infected mice were layered on the top of each sucrose and centrifugated at 10,000xg, 30 min, 4 °C. B – sucrose step gradient centrifugation of the band 2 material. Material recovered from band 2 of the gradient shown in Fig. 1A was washed as described in materials, splitted in equal aliquots and each one layered on top of a 4 ml sucrose step gradient.

On the other hand it was found that material obtained from band 2 was infectious for the mice since it was possible to develop the disease with the same speed and characteristics than equivalent amounts of tachyzoites of the original mouse inoculum (data no shown). In order to further analyze the quality of the purification, isolated DNA from the material found in each band from the first sucrose gradient was spotted onto nitrocellulose sheet (Fig. 3). Only spots from material containing mouse DNA (control mouse DNA, crude tachyzoites preparation, band 1) gave strong signals when hybridized with mouse genomic 32p DNA probe (Fig. 3, MOUSE). On the contrary, when the *T. gondii* 32p DNA probe was used, signals were only detected with the DNA extracted from either purified or crude parasite preparation (Fig. 3, TOXO).

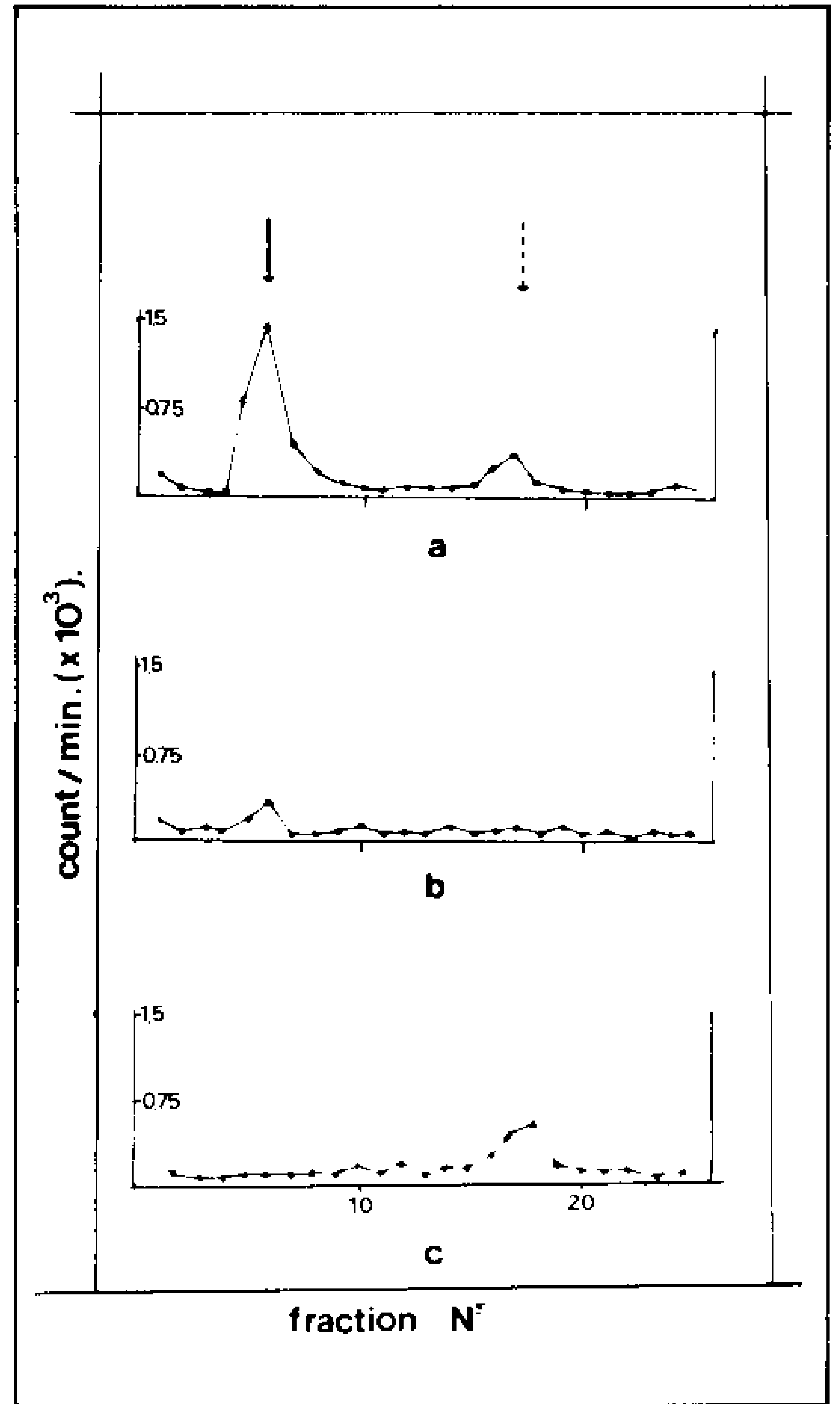

Fig. 2: dot spot hybridization of fractions from sucrose gradients. Gradients were collected in 0.5 ml fraction by puncturing the bottom of the tubes. Fractions from sucrose gradients, containing mouse peritoneal cells or live *Toxoplasma gondii* tachyzoites were spotted onto nitrocellulose filters, lysed *in situ*, and hybridized with a mouse genomic 32p DNA probe (a, b) or with a *T. gondii* genomic 32p DNA probe (c), obtained from band two material of the second sucrose gradient (Fig. 13). After hybridization, spots were cut and their radioactivity was individually measured by Cherenkov effect.

To determine the degree of host cells contamination in each preparation, a dot blot experiment was performed (Fig. 4). Between three or four times the amount of DNA from crude parasite preparations and more than sixteen times the amount of DNA from purified tachyzoites are required to give a signal of the same intensity to that of the mouse liver DNA (Fig. 4, MOUSE). These results indicated that the contamination with host cell DNA represented between 25 to 35% of the genomic material extracted from crude parasite preparation and less than 8% of the DNA prepared

from the purified tachyzoites. When an identical filter was hybridized with a genomic *T. gondii* DNA probe, a similar result was obtained (Fig. 4, TOXO).

Fig. 3: dot blot hybridization with different DNA preparations from the first sucrose gradient. Two quantities (150 and 300 ng of DNA) of DNA from diferent bands of the first sucrose gradient and controls (mouse, *Escherichia coli* and unfractioned parasite preparation DNA) were spotted onto nitrocellulose filters and hybridized with either 32p mouse DNA probe (MOUSE) or 32p *Toxoplasma gondii* genomic DNA probe (TOXO).

In order to perform a preliminary characterization of *T. gondii* genome, genomic DNA of purified parasite preparations was obtained and digested by several restriction endonuclease enzymes (Fig. 5A), showing that the purification procedure did not affect the DNA structure. The absence of cross hybridization between *T. gondii* and mouse genomic DNA in southern experiments confirms the quality of purification (data not shown). DNA endonuclease enzymes that recognize sequences rich in Guanine-Cytosine (like Hap II, Msp I, Hae III, Mbo I and Hind III) produced a high number of clear repetitives sequences (Fig. 5B) indicating an important concentration of those sequences in *T. gondii* DNA. Finally it was interesting to notice that *T. gondii* tachyzoite DNA was totally digested with enzymes that are not active when DNA is methylated (Fig. 5A, lanes Msp I, Hpa II, and Mbo I), in contrast with mouse DNA that contains high levels of methyl cytosine (data not shown). This feature allowed us to obtain a clean isolation of repetitive sequences when digestions of *T. gondii* were carried out with Hap II (Fig. 5B, lane Hap II).

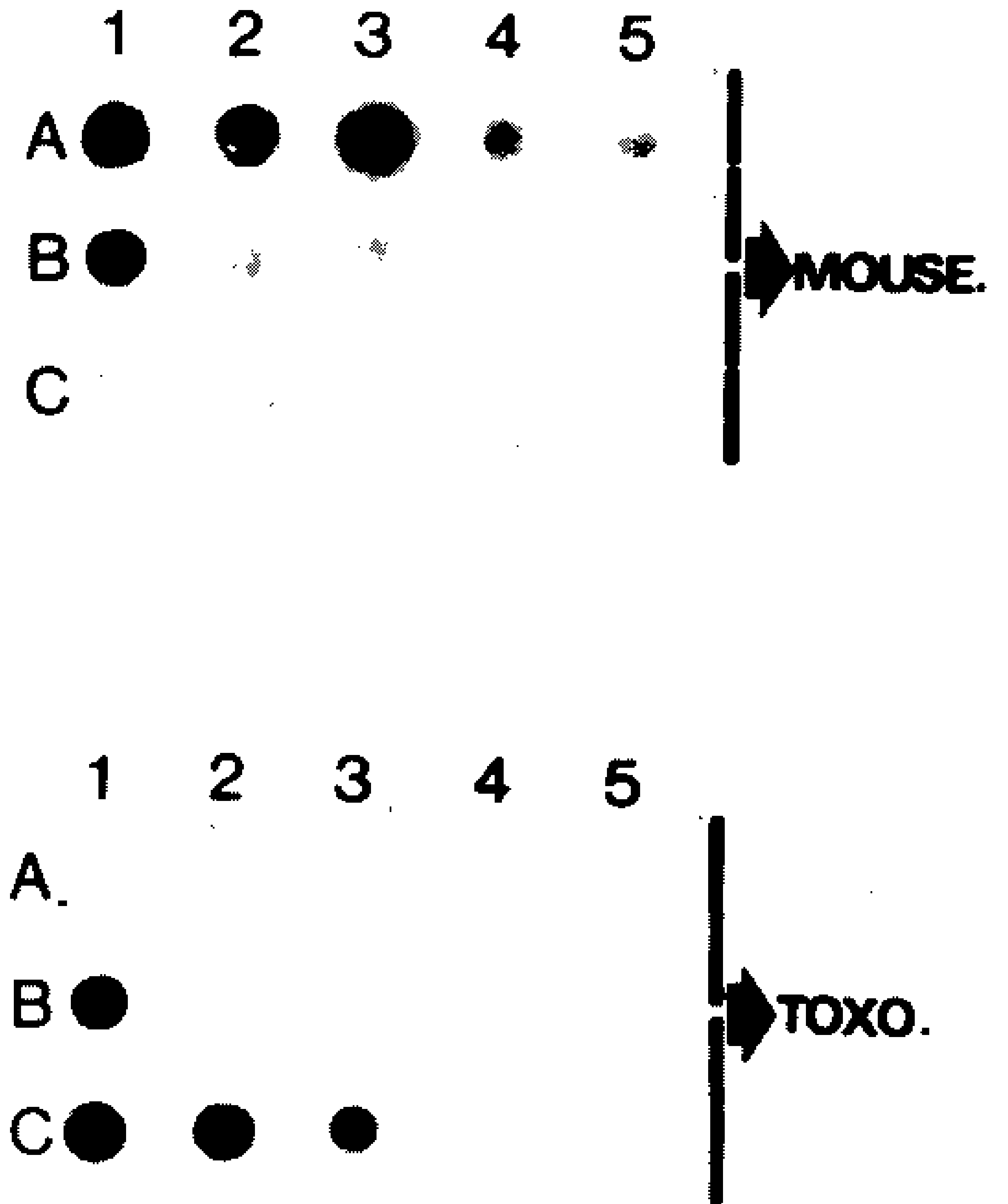

Fig. 4: analysis of the degree of purification of *Toxoplasma gondii* tachyzoites. Two fold dilutions of DNA samples obtained from mouse liver (A), peritoneal exudates of *T. gondii* infected mice (B), and purified *T. gondii* tachyzoite preparations by two successives sucrose gradient (C) were spotted onto nitrocellulose filters and hybridized with either a mouse genomic 32p DNA probe (MOUSE) or a *T. gondii* genomic 32p DNA probe (TOXO). Quantities of DNA were as follow: 1, 200 ng; 2, 100 ng; 3, 50 ng; 4, 25 ng; 6, 12.5 ng.).

DISCUSSION

The availability of large quantities of pure parasite material is a prerequisite for obtaining adequate insight into the biology and physiology of *T. gondii.* Development of specific DNA probes as well as studies about the genetic and molecular biology of the parasite, which are likely to yield knowledge of the host-parasite relationship, requires pure *T. gondii* DNA. A variety of methods to overcome this problem have been proposed. Those consisting in further purification of tachyzoites have failed to produce preparations that are less of 0.5% impure (Tsunematsu, 1960; Masihi et al., 1976).

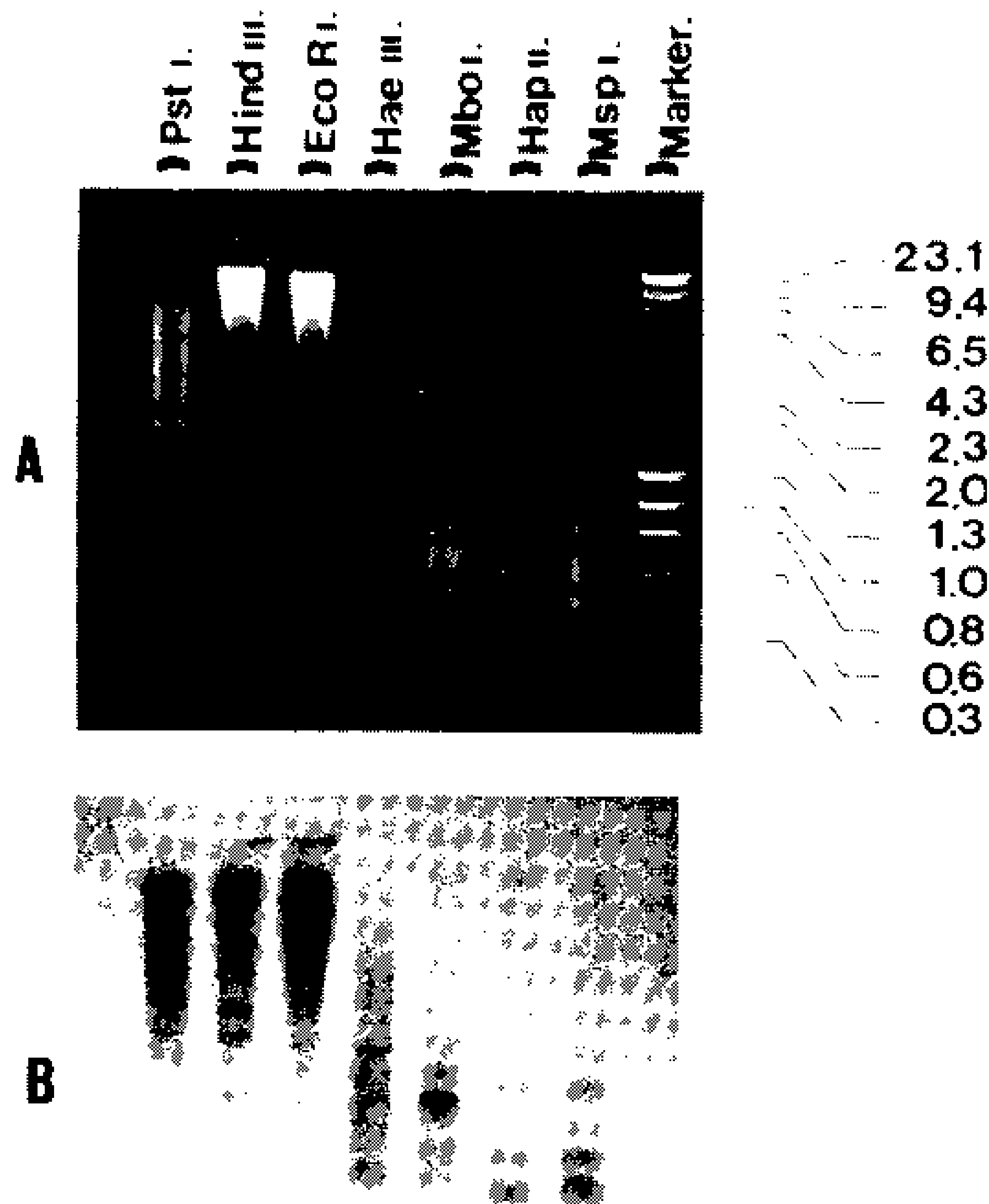

Fig. 5: southern blot analysis. *Toxoplasma gondii* DNA isolated from the material of the second sucrose gradient (Fig. 3 Toxo band) was digested with several endonucleases (EcoRI, MspI, HapII, HindIII, MboI, HaeIII, PstI), electrophoresed on a 1% agarose gel (A), transferred to nitrocellulose and hybridized with a *T. gondii* genomic DNA probe. Autoradiography showing highly repetitive DNA sequences is depicted (B). The markers denote size in kb.

The approach that separates *T. gondii* DNA from murine contamination on the basis of its GC content involves an ultracentrifugation in CsCl Hoeschst 33258 gradient which is both expensive and time consuming. On the contrary we present here a very fast, simple and efficient method for obtaining highly purified *T. gondii* tachyzoites (over 99.9% of purity), which renders higher than 90% of pure DNA preparation. Furthermore, it requires only two low speed centrifugation of 30 min each, to obtain parasite preparation pure enough for biochemical and molecular biology manipulations.

Toxoplasma gondii DNA extracted from purified tachyzoites could be restricted by a variety of partial characterization was assessed by southern blots. These analysis showed clear differences between the DNA repetitive sequences of *T. gondii* and higher eukaryotes, i.e. mouse (data not shown).

In contrast to higher eukaryotic DNAs, where methylation of nucleotides is common, tachyzoites of *T. gondii* seems to have a low level of methylation as it was demonstrated by restriction of the DNA of the parasite with Msp I, Hap II and Mbo I (Fig. 5B). The presence of methylated nucleotides has been related to decreased transcriptional activity (Reeves, 1984) and in this sense it would be interesting to study other stages of the parasite life cycle where transcriptional activity is less active.

The biological relevance of repetitive sequences is up to present not clear. Repetitives sequences are ubiquitous components of eukaryotic genomes. Some classes like satellite DNA, are likely to have an structural role (Brutlag, 1980) whereas others such as the ID sequences of the rat may be involved in gene regulation (Milner et al., 1984). The high prevalence of repetitive sequences in *T. gondii* DNA (especially when enzymes with recognition sequences rich in GC are used) suggests that they might play an important role in the phisiology of the parasite, but their exact function is not yet understood.

Given that cloned DNA repetitive sequences have been found to be excellent tools as DNA probes for taxonomic and diagnostic purposes (Franzen et al., 1984). Those *T. gondii* repetitive sequences described in this work with Hap II restrictive digestion appear ideal for the development of specific DNA probes (Blanco et al., 1990).

Finally it should be emphasized that a combination of the procedure described herein which is very fast for obtaining highly purified tachyzoites with that described by Johnson et al. (1986) for the purification of genomic DNA will allow the obtention of extremely pure DNA for molecular studies. Work in this direction is under progress in our laboratory.

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