

INHIBITION OF GROWTH OF *LEISHMANIA MEXICANA MEXICANA* BY *LEISHMANIA MEXICANA AMAZONENSIS* DURING "IN VITRO" CO-CULTIVATION

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Inhibition of one Leishmania subspecies by exometabolites of another subspecies, a phenomenon not previously reported, is suggested by our recent observations in cell cloning experiments with Leishmania mexicana mexicana and Leishmania mexicana amazonensis. Clones were identified using the technique of schizodeme analysis. The phenomenon observed is clearly relevant to studies of parasite isolation, leishmanial metabolism, cross-immunity and chemotherapy.

Key words: cloning of *Leishmania* – kDNA - schizodeme analysis

During "in vitro" culture leishmanial promastigotes liberate exometabolites or excretion factors that have the capacity to inhibit blastic transformation and enzyme activity in mammalian cells (Schnur et al., 1972; El-On et al., 1979; Walter et al., 1982; Londner et al., 1983).

Here we report a new phenomenon, previously undescribed, the inhibition of growth of one subspecies of *Leishmania* by exometabolites of another subspecies. This phenomenon first appeared in experiments on cell cloning using artificial mixtures of a strain of *L.m. mexicana* and a strain of *L.m. amazonensis* (Pacheco, 1985).

Clones were identified by electrophoretic analysis of fragments generated by restriction enzyme digestion of kinetoplast DNA (kDNA). This technique is also known as schizodeme analysis (Morel et al., 1980).

MATERIALS AND METHODS

Parasite strains – Two strains were used in this study: *Leishmania mexicana amazonensis* strain MHOM/BR/73/M2269 (IOC-L59) and *Leishmania mexicana mexicana* strain MHOM/BZ/58/5 (IOC-L117). These strains have been maintained cryopreserved as well as by "in vivo" serial passage in mice and "in vitro" culture in biphasic medium. The biphasic medium consists of brain-heart infusion agar (Difco), 5.2% (w/v) to which 15% rabbit blood was added. An overlay of enriched brain-heart infusion-liver

infusion-trypticase liquid medium contain 10% heat inactivated fetal bovine serum (Flow Laboratories) was used (Grimaldi, quoted by Jaffe et al., 1984).

Cell cloning – *Leishmaniae* were cloned using the technique of colony formation developed by Jaffe et al. (1984) based on the protocols of Keppel & Janovy (1980) and Tanuri et al. (1981) for the plating of trypanosomatids on solid media. The medium used was brain heart infusion (BHI) – blood agar containing 3.7% (w/v) BHI pH 7.3, 1% (w/v) granulated agar and 10% (v/v) defibrinated rabbit blood.

Promastigotes were counted in a Neubauer chamber and serially diluted (1:10) in PBS pH 7.3 so that a minimum of 10 and a maximum of 40 cells per 100 μ l were plated. The cellular suspension was spread with a Drigalski loop on the surface of the plates which were sealed and incubated at 25°C in conditions of constant humidity.

The resulting colonies were retrieved intact, one by one, from the surface of the plates by aspiration using a Pasteur pipette and then transferred to tubes with culture medium. Stocks obtained from the clones were inoculated to obtain the cell mass for kDNA extraction.

Extraction of kDNA and digestion with restriction Enzyme – For the rapid extraction kDNA (Gonçalves et al., 1984 b) the cell mass with about 10^9 promastigotes was resuspended in SE 1X (0.15 M NaCl, 0.1 M EDTA pH 8.0) and lysed with a strong detergent (sarkosyl) and digested by pronase at 60°C. The kDNA networks were collected by centrifugation, extracted by phenol-chloroform and precipitated by ethanol. The total quantity of kDNA was calculated as follows: 1 unit of absorbance at 260nm corresponds to 50 μ g of kDNA/ml.

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The digestion of kDNA preparations with restriction enzyme was carried out at 37°C during 3 hours by adding to 2 µg of kDNA, 2U of restriction enzyme Bsp RI and the appropriate reaction buffer. The reaction was interrupted by addition of 2 µl of an indicator solution (0.05% xylene cyanol, 0.05% bromophenol blue, 50% glycerol, 5% sarkosyl).

The electrophoresis was carried out in a linear polyacrylamide gradient gel of 4.5% to 10% at 10 mA overnight. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light (300nm) or by the technique of silver staining (Gonçalves et al., 1984 b) and photographed under a normal fluorescent light transilluminator.

RESULTS

When equal numbers of promastigotes of stocks MHOM/BZ/58/5 (IOC-L117) and MHOM/BR/73/M2269 (IOC-L59) from individual culture tubes were mixed and plated together all the resulting colonies were identified as *L. m. amazonensis* (IOC-L59). Colonies of each subspecies appeared in the expected numbers when plated individually on control plates. In the first experiment 12 promastigotes from a 6-day culture of stock IOC-L117 of *L. m. mexicana* was mixed with 12 promastigotes of a 6-day culture of stock IOC-L59 of *L. m. amazonensis*. After 8 days of incubation there were 6 isolated colonies on the plate; however only 4 clones were analysed, as two were lost because of contamination. Fig. 1 shows the restriction enzyme profile of each clone after digestion with the enzyme Bsp RI. All the clones possess profiles similar to *L. m. amazonensis*.

These results were confirmed in a second experiment. The two subspecies were co-cultivated with equal numbers of cells in biphasic medium during 6 days and after time an aliquot of the mixed culture was diluted to a concentration of 20 promastigotes/100 µl and plated. The results are shown in Fig. 2 where only profiles similar to *L. m. amazonensis* are observed in the 10 clones analysed. In contrast to these results the analysis of a mixed culture after 6 days of growth clearly shows a mixed profile (Fig. 2 channel 13) demonstrating a superposition of the profile of the 2 subspecies. Fig. 3 shows the similarity between growth curves and generation times of the two parasites in biphasic medium. The third experiment was carried out in order to investigate the hypothesis that some factor produced by the dominant subspecies and excreted in the medium was interfering in the growth of the subspecies. In this experiment the supernatant from a 20 day old culture of *L. m. amazonensis* was fil-

tered through a Millipore filter and spread on the surface of 3 plates of solid media. On one of the plates *L. m. amazonensis* was inoculated, on the other plate *L. m. mexicana* and on the third plate a mixture of the two subspecies. Two control plates without the supernatant were also inoculated.

In all the plates colonies were formed after 8 days of culture with the exception of the plate containing *L. m. mexicana* which remained negative during 20 days of incubation (Fig. 4). An artificial mixture of 1 µg of kDNA/ µl of each of the stocks (channel 14) reproduced the restriction profile obtained from the analysis of the mixed culture of 6 days growth.

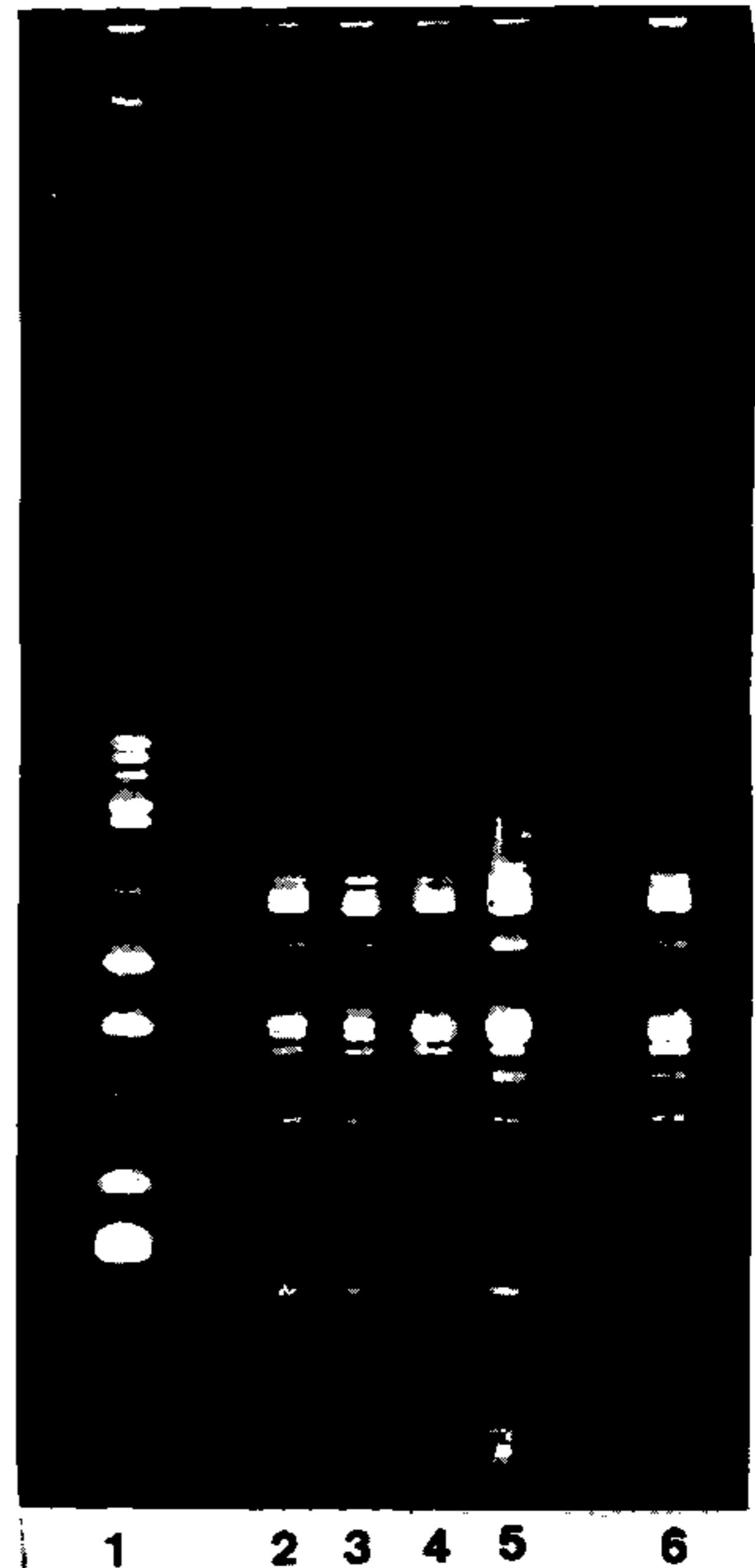


Fig. 1: Electrophoresis in a polyacrylamide gradient gel (4.5%-10%) showing fragments of kDNA after digestion with the restriction enzyme Bsp RI (stained by ethidium bromide) - 1) *L. m. mexicana* (IOC-L117); 2-5) clones of the mixture; 6) *L. m. amazonensis* (IOC-L59).

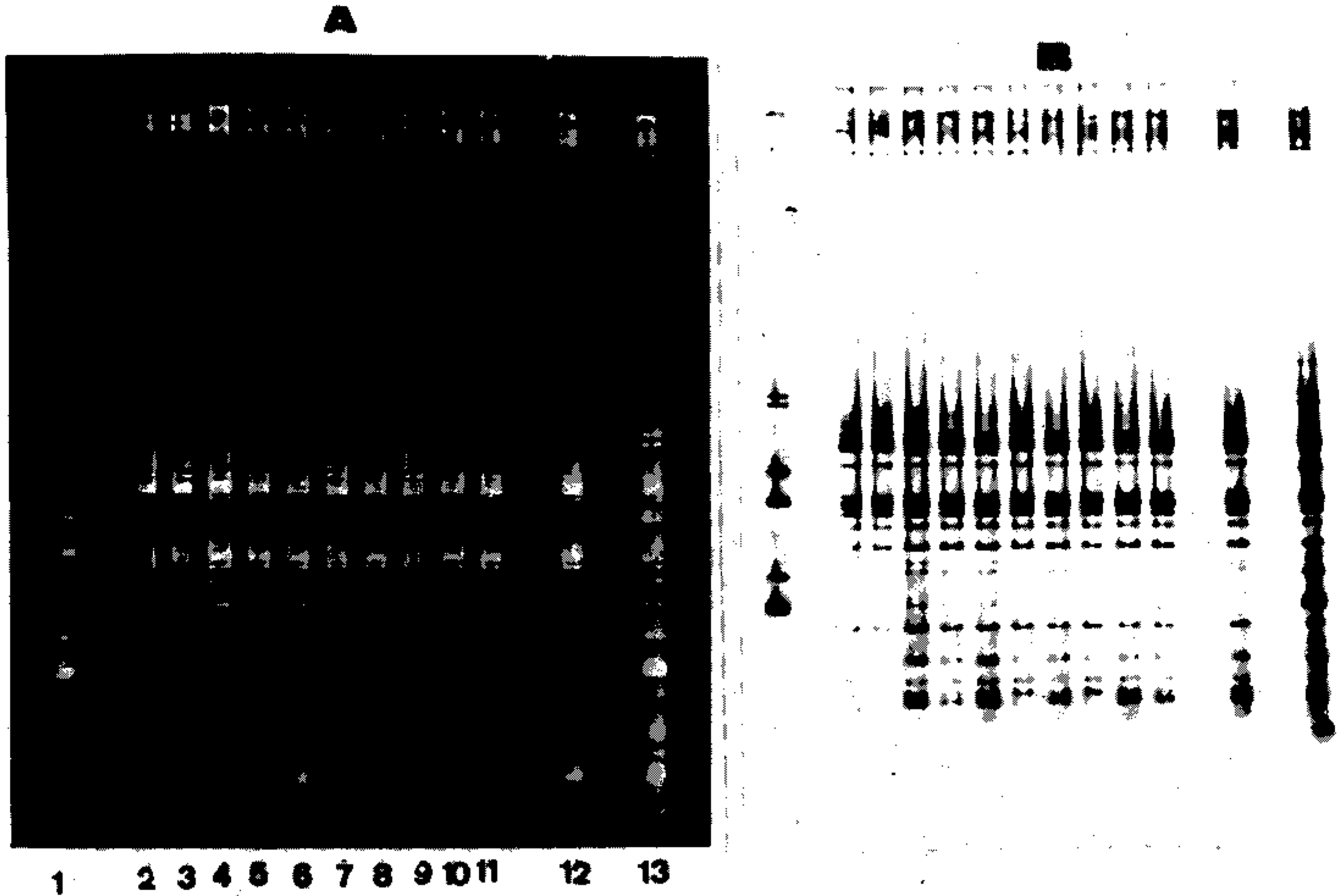


Fig. 2: Photograph of a polyacrylamide gel showing the Bsp RI schizodeme analysis of each clone (Fig. 2A: stained by ethidium bromide, Fig. 2B: stained by silver nitrate) – 1) *L.m. mexicana* (IOC-L117); 2-11) clones of the mixture; 12) *L.m. amazonensis* (IOC-L59); 13) restriction profile of the 6 day-old mixed culture.

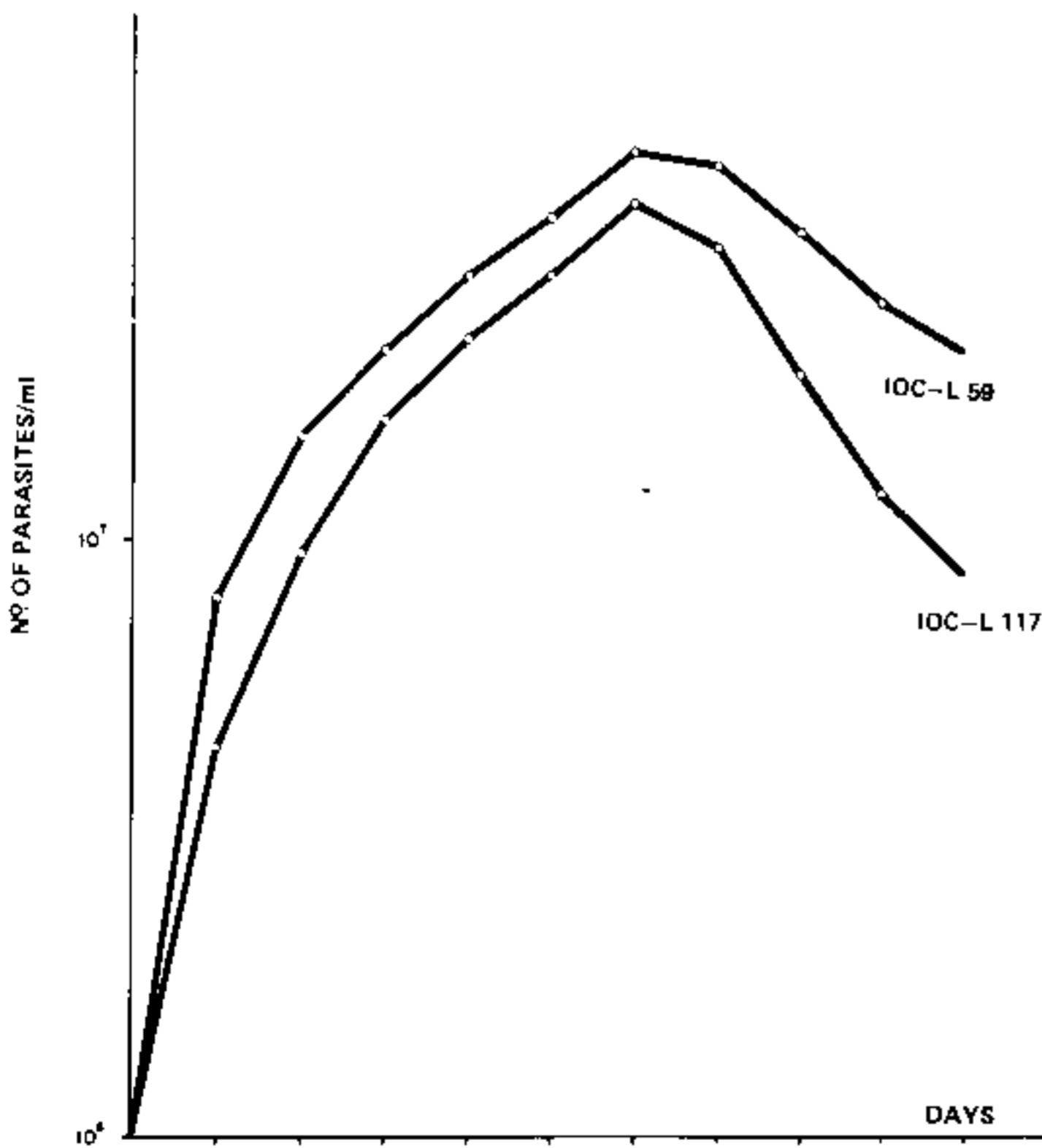


Fig. 3: Growth curve of *L.m. mexicana* (IOC-L117) and *L.m. amazonensis* (IOC-L59) in biphasic medium. Each point represents the mean of three measures. The generation time calculated for the stocks IOC-L117 and IOC-L59, respectively, was: 27hr 30 min and 26hr 30 min.

The procedure used in the 4th experiment was basically the same as before except that sterilized supernatant was used from a 20-day culture of *L.m. mexicana*. Parallel to this a 12-day culture of 2 parasites in biphasic medium was analysed. *L.m. amazonensis* grew in the presence of the heterologous supernatant; however the opposite did not occur in the case of *L.m. mexicana* as shown in Fig. 5. All the clones from the plates inoculated with a mixture of the 2 parasites and containing *L.m. mexicana* supernatant were identified as *L.m. amazonensis* (channel 5 to 12). Analysis of the mixed culture from the 12-days culture shows a restriction profile compatible with *L.m. amazonensis* instead of a mixed profile.

The last experiment of this series was carried out to investigate the hypothesis that inhibition would not occur if the promastigotes were washed, and free from any interfering factor present in the biphasic culture medium, before dilution and plating.

The results however did not confirm this hypothesis and all the clones were identified as *L.m. amazonensis* (Fig. 6).

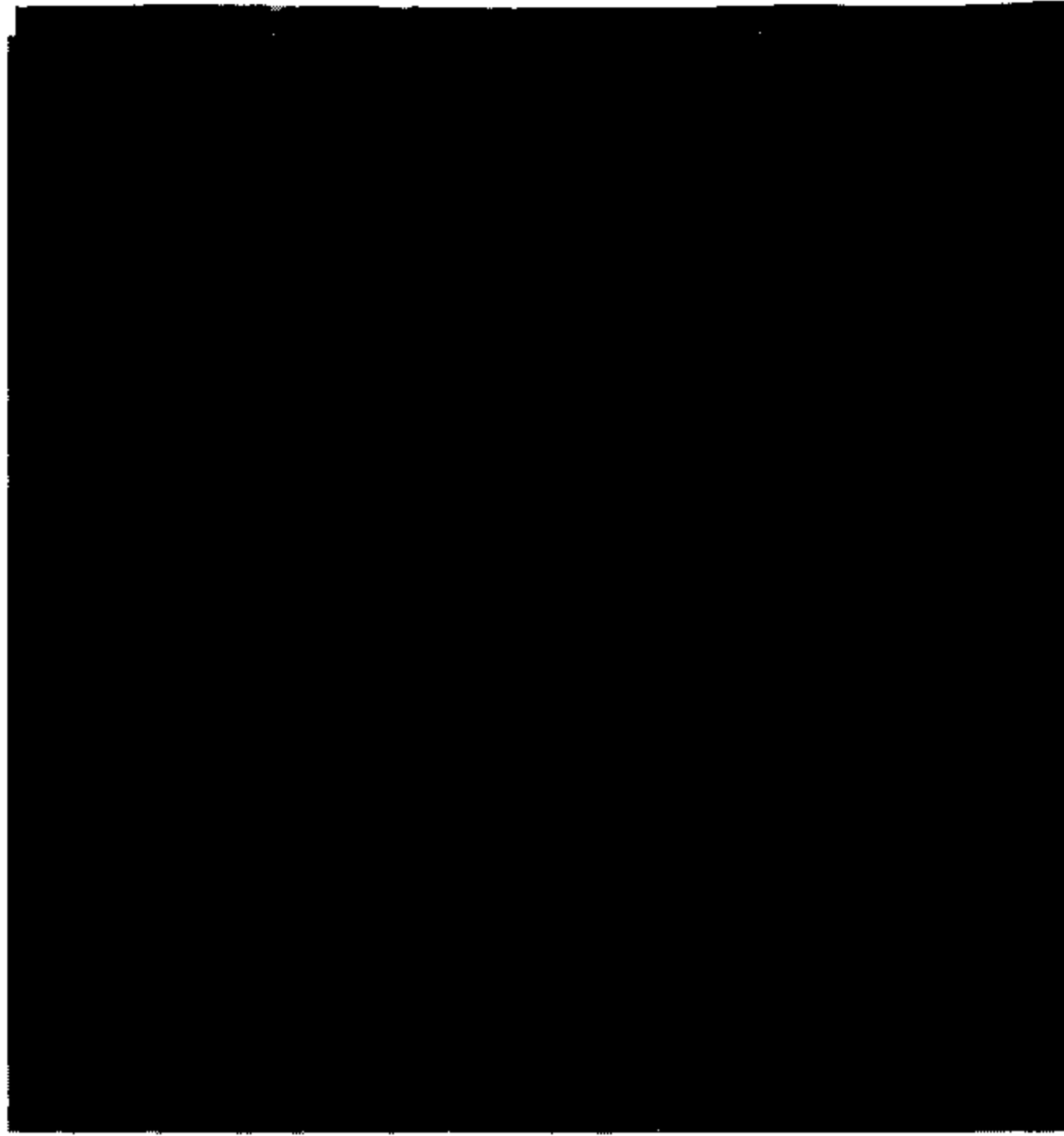


Fig. 4: Photograph of a polyacrylamide gel showing the kDNA restriction profile of each clone – 1-2) *L.m. mexicana* clones (control); 3-4) *L.m. amazonensis* clones (control); 5-9) clones of *L.m. amazonensis* in the presence of the homologous supernatant; 10-13) clones from the mixture of the two subspecies in the presence of *L.m. amazonensis* supernatant; 14) artificial mixture of 1 μg kDNA/μl of each stock (IOC-L117 and IOC-L59).



Fig. 5: Schizodeme analysis showing the kDNA profile of each clone – 1-2) *L.m. mexicana* clones (control); 3-4) *L.m. amazonensis* clones (control); 5-12) clones from the mixture of two subspecies in the presence of *L.m. mexicana* supernatant; 13-14) clones of *L.m. amazonensis* in the presence of heterologous supernatant; 15-16) clones of *L.m. mexicana* in the presence of homologous supernatant; 17) restriction profile of the 12 day-old mixed culture.

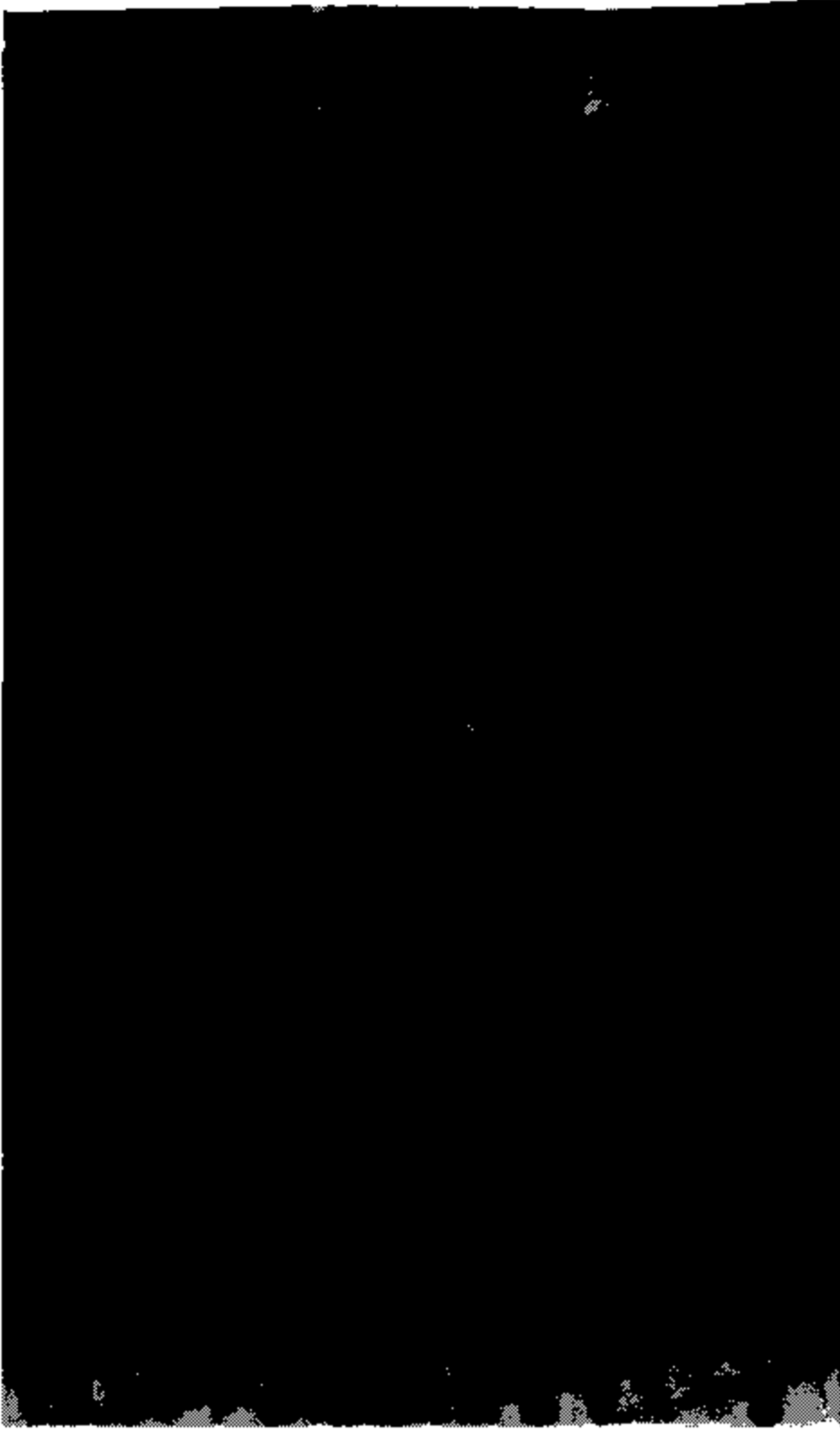


Fig. 6: Electrophoretic analysis of the kDNA fragments generated after digestion with the restriction enzyme Bsp RI – 1) *L.m. amazonensis* (IOC-L59); 2-7) clones of the mixture; 8) *L.m. mexicana* (IOC-L117).

DISCUSSION

In five experiments of cloning using a mixture of promastigotes from strains of *L.m. amazonensis* and *L.m. mexicana* and carried out under different conditions, the same result was obtained. That is, there was a total predominance of *L.m. amazonensis* among the clones analysed. Although 3 different schizodemes were identified among the clones produced from this stock, there was no case where the profile could be interpreted as resulting from a mixture between the 2 strains. According to Gonçalves et al. (1984 a) studies with *Trypanosoma cruzi* showed that schizodeme analysis is capable of detecting a mixture of 2 strains with different profiles even in the proportion of 1:99.

In contrast to our results during co-cultivation in solid media the culture of equal number of promastigotes from the 2 strains in biphasic media did not result in the complete inhibition of *L.m. mexicana* during 6 days of growth. The kDNA profile obtained included bands representing the 2 subspecies; however after 12 days of culture, only the profile of *L.m. amazonensis* was identified.

Assuming that the 2 subspecies grow well in solid media and require the same period of time to form colonies as when they are independently plated in the control experiments, and that the growth curve and the generation time of the 2 parasites are similar, the possibility of metabolic competition becomes remote. On the other hand, it would be a generalization if we affirmed that this phenomenon only occurs in solid media.

It is therefore possible that some product excreted by the dominant strain and when in sufficient concentration exercises an inhibitory action. However the possibility should be considered that this effect is dose-dependent. One of the experiments shows the effect produced by the supernatant of an old culture of *L.m. amazonensis* on the growth of *L.m. mexicana*. The opposite effect however is not seen. That is, the heterologous supernatant appears to have no effect on the growth of *L.m. amazonensis*. The last experiment reinforces the idea that there is an inhibitory factor that is constantly produced and excreted by the promastigote forms during growth.

We do not have at the moment further explanations for the phenomenon observed and its mechanism of action. However our results indicate that a metabolic competition between the two strains is not involved and suggest that *L.m. amazonensis* produces substances that inhibit the growth of *L.m. mexicana* "in vitro".

Additional experiments will be necessary to elucidate the mechanism of this phenomenon, including biochemical and immunological "in vitro" and "in vivo" studies due to its importance in investigations of metabolism, cross-immunity and chemotherapy.

RESUMO

Inibição do crescimento de *Leishmania mexicana mexicana* por *Leishmania mexicana amazonensis* durante o co-cultivo "in vitro" – Inibição do crescimento de um subespécie de *Leishmania* por exometabólitos de outra subespécie, um fenômeno ainda não notificado, é sugerido em nossas recentes observações em experimentos de clonagem celular com *Leishmania mexicana mexicana* e *Leishmania mexicana amazonensis*. Os clones foram identificados usando a técnica de análise de esquizodemas. O fenômeno observado é claramente relevante em estudos de isolamento parasitário, metabolismo, imunidade cruzada e quimioterapia.

Palavras-chave: clonagem de *Leishmania* – kDNA – análise de esquizodemas

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