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FLUOROGENIC DETECTION OF VIABLE *TOXOPLASMA GONDII*

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Summary :

In order to easily assess growth and destruction of *Toxoplasma gondii in vitro*, this report describes two double staining assays that both visualize live and dead organisms: acridine orange - ethidium bromide (AO-EB) and bisbenzimide (Hoechst 33258) - propidium iodide (B-PI). EB and PI were chosen for dead organisms staining while AO and B stain viable organisms. Thus, both double staining assays seem more informative than Giemsa staining or indirect immunofluorescence. They offer methods to study internal structure of the parasite as well as information on host-parasite relationships. Moreover, detection in culture are sensitive, easier, and less time consuming than previous methods. So, they should to be useful in strains behaviour analysis.

KEY WORDS : *Toxoplasma gondii*, acridine orange, bisbenzimide, viability.

Résumé : DETECTION FLUOROGÉNIQUE DE *TOXOPLASMA GONDII* VIVANTS

Afin d'évaluer facilement la multiplication et la destruction de Toxoplasma gondii *in vitro, deux doubles colorations sont décrites. Les associations acridine orange – bromure d'éthidium et bisbenzimide (Hoechst 33258) – iodure de propidium permettent de visualiser les organismes vivants et morts. En effet, le bromure d'éthidium et l'iodure de propidium ont été choisis pour colorer les organismes morts alors que l'acridine orange et le bisbenzimide l'ont été pour colorer les organismes vivants. Ces associations semblent plus informatives que le Giemsa et l'immunofluorescence indirecte et vont de plus mettre en évidence les différentes structures internes du parasite ainsi que ses relations avec la cellule hôte dont la viabilité pourra également être étudiée. Ces techniques sont plus faciles et plus rapides à mettre en œuvre ; elles permettent d'apprécier la viabilité du parasite en culture ou dans des milieux biologiques ainsi que le comportement de différentes souches de ce parasite* in vitro.

MOTS CLÉS : Toxoplasma gondii, *acridine orange, bisbenzimide, viabilité*

Toxoplasma gondii is an intracellular protozoan parasite that usually causes an asymptomatic or a benign self-limiting infection, but may induce a severe disease in immunocompromised patients or for the foetus. Culture methods are widely used to detect *T. gondii* in human samples. The detection of the parasite is mostly performed by using direct examination (Giemsa), indirect immunofluorescence or a radioassay. Nevertheless, the correct status of the infection can only be determined by the identification of viable parasites. Acridine orange alone provides simultaneous information concerning cell viability as well as nuclear morphologic characteristics. Some investigators used acridine orange in the determination of *T. gondii* viability before challenge (Marin *et al*, 1996; Pelloux *et al*, 1996; Kaneshiro *et al*, 1993; Sharma *et al*, 1986). Acridine orange has been used to stain numerous parasites, including *Plasmodium* (Hensen *et al*, 1970, Kawamoto *et al*, 1991, Lowe *et al*, 1996), *Pneumocystis carinii* (Thomson & Smith, 1986), *Blastocystis hominis* (Suresh *et al*, 1994), *T. gondii* (Sharma *et al*, 1986), and intracellular bacteria in culture or clinical specimen (Heinzen *et al*, 1996). The fluorochrome dye bisbenzimide 33258 Hoechst is widely used for *Mycoplasma* and *Pneumocystis carinii* detection and stain viable organisms (Laube *et al*, 1997). The intercalating dyes, ethidium bromide and propidium iodide, are known to pass only through the membranes of dead or dying cells. Thus, in order to differentiate dead or living parasites within infected cells, we report here the use of two associations of two fluorochrome dyes [acridine orange/ethidium bromide (AO-EB) and bisbenzimide/propidium iodide (B-PI)] to study the multiplication ability of trophozoites of different isolated *T. gondii* strains and their destruction by the host cells in culture.

The RH strain of *T. gondii* was obtained from peritoneal fluid of mice. Strains were initially isolated from human sample and cultured on the human myelomonocytic cell line unstimulated THP1 (ECACC n° 88081201) at 37 °C in a humidified atmosphere containing 5 % CO_2 in RPMI medium supplemented with 10 % heat decomplemented foetal calve serum, 2 mM glutamine. 500 µl aliquot of parasited cells were removed from a stock culture. 10 µl AO-EB (100 mg/ml)

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or 10 µl B-PI (10 µg/ml) were added. After one minute incubation, infected cells were observed with an Axioscop microscope (Zeiss, Germany), using a BP490 green filter (excitation 480 nm / emission 520 nm for AO-EB and excitation 330 nm / emission 380 nm for B-PI). The observations were made at 400× magnification. Number of cells containing tachyzoites and tachyzoite number in the parasitophorous vacuoles were estimated.

AO-EB procedure identified non viable cells with nuclei rapidly stained in red by EB; live host cell cytoplasm remained dark green with nuclei bright green fluorescence (Fig. 1 & 2). Live tachyzoites showed a dark green cytoplasm whereas dead ones showed light orange-green cytoplasm fluorescence (Fig. 2). The B-PI stain revealed that live cells presented a blue fluorescence, whereas non viable organisms showed pink fluorescence (Figs. 3 & 4). Viable forms and replicating parasites appeared with blue clumping nuclei (Fig. 3). Intra- and extra-cellular viable and degenerating *Toxoplasma* trophozoites were seen in the first hour after the challenge.

The aspect of parasites observed with double staining assays is more informative than using conventional methods. Moreover parasites and cells viability is evaluated and internal structures are seen. These differences are due both to the selective staining characteristics of fluorogenic substrates and to the greater resolution afforded by the ultraviolet excitation of stained structures. AO-EB staining seems more informative and easier to read than B-PI staining or AO-PI staining (results not shown). In addition, fluorochrome double staining methods may be useful for studying the diffe-

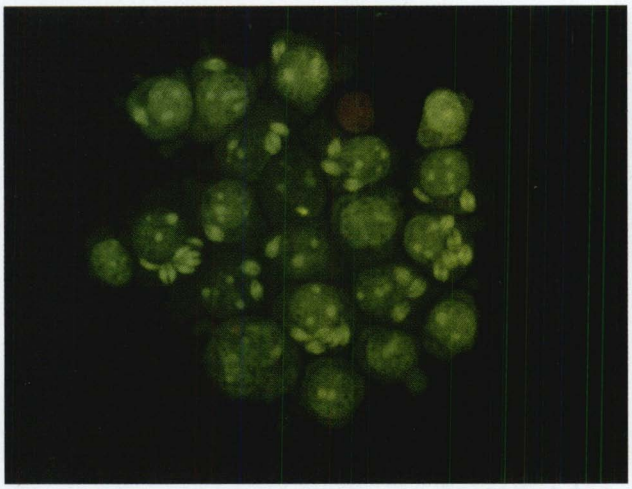

Fig. 1. – THP1 cells containing viable *T. gondii* organisms [acridine orange-ethidium bromide stains (× 400)].

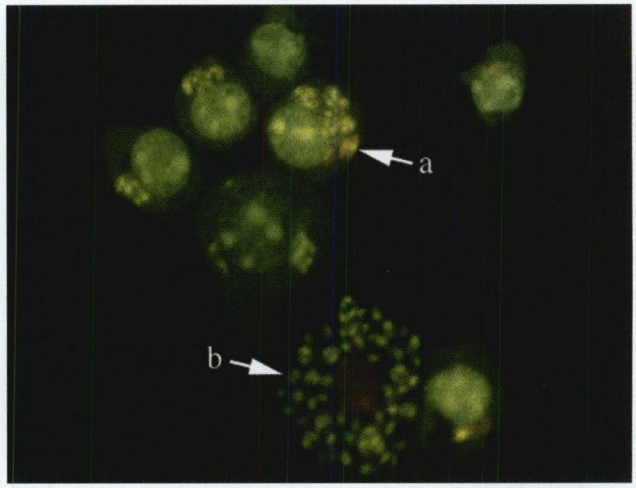

Fig. 2. – Acridine orange-propidium iodide stains (× 400): Arrow a: dead intracellular *T. gondii* organisms with bright orange cytoplasm; Arrow b: viable tachyzoites in a dead THP1 cell.

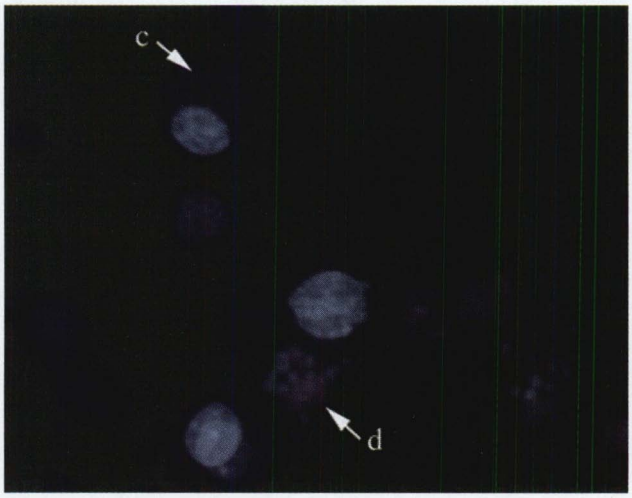

Fig. 3. – Bisbenzimide-propidium iodide stains (× 400): Arrow c: viable *T. gondii* organisms in viable THP1 cell; Arrow d: viable *T. gondii* organisms in a dead THP1 cell.

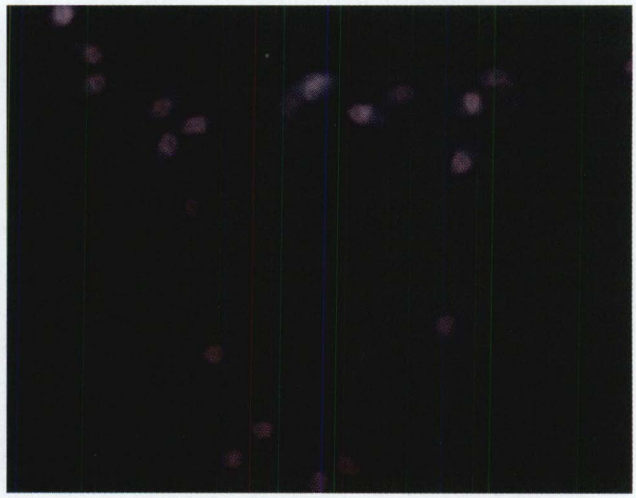

Fig. 4. – Dead free tachyzoites with pink nuclear [bisbenzimide-propidium iodide stains (× 400)].

rent strain behaviour *in vitro* and virulence phenotype expression. Indeed, double stains allow identification of viable free and intracellular organisms (cysts, free tachyzoites) in a different manner than dead and non replicating viable organisms. Then this double stain shows the host cell ability to kill intracellular pathogens.
Also, since drug screening in infected animals and radioassay is time consuming and expensive, *in vitro* viability assays should have applications in studies on drug development against protozoa responsible for human disease.

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