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QUANTITATIVE *Toxoplasma gondii* OOCYST DETECTION BY A MODIFIED KATO KATZ TEST USING KINYOUN STAINING (KKK) IN ME49 STRAIN EXPERIMENTALLY INFECTED CATS

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SUMMARY

We detected *Toxoplasma gondii* oocysts in feces of experimentally infected cats, using a Kato Katz approach with subsequent Kinyoun staining. Animals serologically negative to *T. gondii* were infected orally with $5x10^2$ mice brain cysts of ME49 strain. Feces were collected daily from the 3rd to the 30th day after challenge. Oocysts were detected by qualitative sugar flotation and the quantitative modified Kato Katz stained by Kinyoun (KKK). In the experimentally infected cats, oocysts were detected from the 7th to 15th day through sugar flotation technique, but oocysts were found in KKK from the 6th to 16th day, being sensitive for a larger period, with permanent documentation. The peak of oocysts excretion occurred between the 8th to 11th days after challenge, before any serological positive result. KKK could be used in the screening and quantification of oocysts excretion in feces of suspected animals, with reduced handling of infective material, decreasing the possibility of environmental and operator contamination.

KEYWORDS: *Toxoplasma gondii*; Kato Katz; Kinyoun; Oocysts; Cat; Diagnosis.

INTRODUCTION

Humans become infected with *Toxoplasma gondii* mainly by ingesting uncooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts from the feces of infected cats (DUBEY & BEATTIE, 1988). *T. gondii* oocysts are shed in large numbers by domestic cats and other members of the *Felidae* after ingesting prey or contaminated water (HILL & DUBEY, 2002). These oocysts mature in the environment and are disseminated through rain and surface water, resulting in widespread contamination of the environment (DUBEY & FRENKEL, 1972; DUBEY, 2001). Kittens are probably the major source of contamination as they are common and produce large numbers of oocysts (DUBEY & CARPENTER, 1993).

Waterborne outbreaks of acute toxoplasmosis worldwide reinforce the transmission of *T. gondii* to humans through water contaminated with oocysts and may have a greater epidemiological impact than previously believed (DUBEY & CARPENTER, 1993; KARANIS *et al., 2007).* Findings regarding the prevalence of *Toxoplasma* oocysts in water are still rare and difficult (BOWIE *et al*., 1997; ISAAC-RENTON *et al*., 1998; DE MOURA *et al*., 2006). Recently, new alternative methods have been proposed, but they involve numerous centrifuging and expensive reagents with molecular detection of *Toxoplasma* DNA (DUMETRE & DARDE, 2004; KOURENTI & KARANIS, 2006). For epidemiologic surveys, seroprevalence in cats allows indirect estimation and are more feasible than oocyst fecal examination, providing an indicator of environmental contamination (MEIRELES *et al.,* 2004). Ooccysts can be detected by examination of cat feces by concentration methods such as flotation in high-density sucrose solution, with risk of environmental and operator contamination due to extensive manipulation (DUBEY, 2004). Routine *Cryptosporidium* oocysts isolation in human feces uses a rapid modified-acid method, Kinyoun stain (AMATO NETO *et al*., 1996), which allows clear identification of the infection with minimum of stool manipulation, but without quantification, which is a feature of the Kato-Katz test (KATZ *et al*., 1972). We studied oocysts excretion in feces of experimentally infected cats by a combination of these methods, by Kinyoun staining of a thick quantitative smear after Kato-Katz, for detection and quantification.

MATERIAL AND METHODS

For experimental cat infection, recently weaned cats were fed with $5x10^2$ cysts of ME-49 strain of *T. gondii* obtained from brains of previously inbred mice (HIRAMOTO *et al*., 2002). The animals were maintained at the Institute of Tropical Medicine of São Paulo University, receiving commercial food and water *ad libitum* and their feces were daily collected to the 30th day post-challenge, with weekly bleeding under anesthesia. The cats were isolated and manipulated before or during the infection according to 'Principles of Laboratory Animal Care' (CLARK, 1996). Feces were examined daily by two methods.

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Sugar flotation technique (SHEATHER, 1923) was performed in 5 g of feces mixed with 45 mL of a sugar solution (density 1.208) and centrifuged at 1,000 *g* for 10 minutes, and the surface film transferred to a slide. Oocyst search was performed by two independent observers, looking for 10 µm thick walled structures, containing cellular structures, as there is no maturation to sporozoites at this stage.

Feces were also processed by a modified Kato Katz stool collection, with subsequent Kinyoun staining (KKK). Briefly, a small amount of fecal material was placed on scrap paper and a piece of nylon sieve was pressed on top of it so that some of the feces sieved through the screen and accumulated on top of the 180 mesh nylon sieve. A spatula was scraped across the upper surface of the screen to collect the sieved feces. The spatula was used to deposit the feces in the orifice of the perforated plate on a microscope slide. The perpendicular 4x10x1 mm orifice was devised to contain exactly 0.04 g or 40 µL of feces, at one corner of the slide. The other side of the spatula was passed over a nylon sieve and over the perforated plate to remove excess feces. The plate was carefully removed by lifting, leaving behind a small square mould of sieved material. Opposite corner of other microscope slide was pressed on top of this, and a relatively thin smear was obtained by sliding over the slides in order to provide a thick smear in each slide. The material was air dried, heated and stained by Kinyoun method as described elsewhere (AMATO NETO *et al*., 1996), with subsequent mounting. *T.gondii* oocysts appeared as red cystic structures with 10 µm diameter, with preservation of internal details. Oocyst frequency in the preparation was determined both by using a quick semi-quantitative method, scoring as (–) when no oocysts were found, (+) when at least one stained oocyst was found in the slide; (++) when rare stained oocysts were found in the slide but not in every field; (+++) when at least one stained oocyst was found in each 20X optical field and (++++) when more than one stained oocyst were found in each 20X optical field. This analysis was easily performed in only one of the KKK slides by two independent observers. Quantitative determination was performed in both slides from each preparation for determination of total numbers of oocysts by two independent observers and the oocysts number per gram of feces determined assuming that the whole preparation in the two slides had 0.04 g of feces.

Serum specific anti *T. gondii* IgG was determined in weekly collected serum samples by a conventional ELISA, using microplate coated with saline extract from RH strain tachyzoites as elsewhere described (MEIRELES *et al*., 2004) and expressed as titer, the inverse dilution of sera that gives 1.0 O.D. over control in the ELISA assay.

RESULTS

Oocyst identification by both methods is demonstrated in Fig. 1. Sucrose flotation allows demonstration of cystic structures, with 10 µm diameter clearly identified by contract phase microscopy, as shown in Fig. 1A. The Kato-Katz-Kinyoun (KKK) shows a clearly identified red structure, over a background of fecal contents, without any other acid fast stained structure, as could be seen in Fig. 1B, allowing also both identification and quantification.

Detection of *T. gondii* oocysts in the stools of four experimentally infected cats (two males and two females) fed with ME-49 *T. gondii* strains is shown in Fig. 2, associated to specific IgG titres. Oocysts

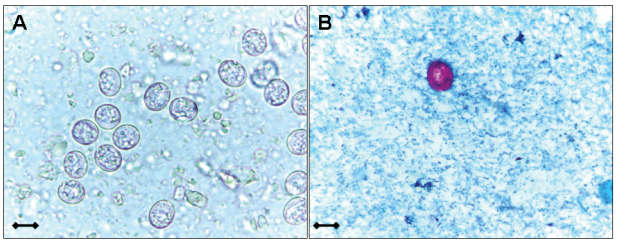

Fig. 1 - Representative images from stool detection of *T. gondii* immature oocysts. A - *T. gondii* oocysts in sucrose solution suspension. B - *T. gondii* oocyst stained by KKK. Bar = 10 µm.

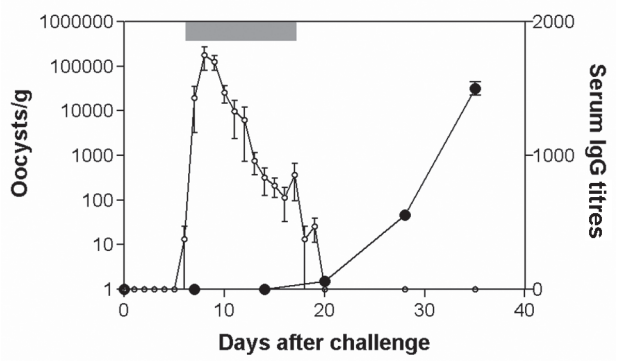

Fig. 2 - Quantitative mean oocyst excretion in cats experimentally infected with *T. gondii* ME-49 strain. Open dots: oocyst/g stools by KKK. Closed dots: mean anti *T. gondii* IgG antibody titres by ELISA. Shaded bar: period of oocyst detection by sucrose flotation. Bars represent SEM (Standard error of mean).

were detected from the 7th to 15th day through flotation technique, but KKK allowed detection of oocysts from the 6th to 16th day, with permanent documentation. Antibody titers were found in sera only after the declining of oocyst excretion which completely disappears after the 17th day of challenge in all animals. The excretion was absent after this period until the 30th day after challenge.

Oocyst excretion in individual animals are shown in Fig. 3, compared to KKK semi-quantitative estimation of oocysts, showing that the peak of excretion occurred in the 8th to 11th days after challenge in all individual animals. Quantitative analysis shows an early acute peak in all animals, with subsequent relatively slow decline, with an asymmetric profile, by both quantitative and semi-quantitative data. The oocyst excretion was very similar in all animals, with all cats showing at least one day with $>10^5$ oocysts/gram of feces. There is a very good association between the semi-quantitative scoring in one slide and the quantitative data, allowing the use of this quick scoring system in the screening of samples for posterior study.

DISCUSSION

Toxoplasma oocysts identification is easily performed with our modification of Kato-Katz direct fecal examination test, allowing quick identification of the presence of oocyst with little equipment. Oocyst preservation in KKK also allows gross genera identification, due to the preservation of oocyst diameter, but more accurate studies other than morphology must be performed for adequate speciation, as elsewhere described (SHARES *et al*., 2005). Without staining, the dried

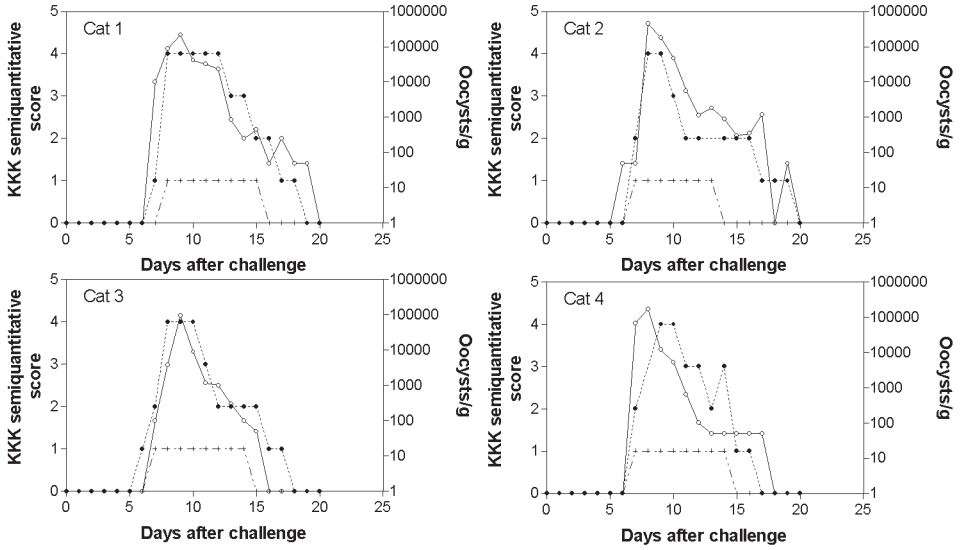

Fig. 3 - Quantitative stool detection of *T.gondii* oocysts in individual experimentally infected cats, as compared by three approaches: Open circles: KKK quantitative determination. Closed circles = KKK semi-quantitative scoring. Dashed line = sucrose flotation findings (0 = negative + = positive).

slide could be an alternative for nucleic acid preservation for genomic speciation, which could be performed by nucleic acid purification and adequate primers in PCR (SALANT *et al*., 2007).

KKK involves few steps of manipulation of infective material, decreasing the possibility of environmental and operator contamination, destroying all viability of the oocysts. The sugar flotation technique has the disadvantage of multiple steps with infective material manipulation and centrifugation, increasing the risk of contamination. KKK also allow quantitative data to be read easily in field conditions, or transported without preservation due to the stable documentation, without new steps required for quantitative determination with sugar flotation. Other advantages of KKK include the small amount of feces required to perform the test as well as the low cost of the technique, together with its suitability for rapid use under field conditions, as the veterinary office.

Oocyst excretion in stools in our experimentally infected cats was short lived, with positive serology occurring after the main excretion of oocysts. These findings were reported elsewhere, despite some descriptions of persistent excretion in some models, ascribed to strains or parasite variation (DUBEY, 2005). Specific IgG serology only becomes evident after oocyst excretion, which results in poor diagnostic value for individual felids that are implicated as a source of *T. gondii* spreading, but it is a feasible technique for feline toxoplasmosis prevalence, with results related to a past excretion of oocysts and chronic infection (MEIRELES *et al*., 2004, SALANT *et al*., 2007).

Morphological approaches on *Toxoplasma* oocysts identification have been neglected in recent years, due to the upsurge of more precise technologies, but could be also useful for veterinary practice, for diagnosis or screening sick animals. We suggest that this modified technique could be introduced for screening and detection of oocyst excretion in feces of suspected animals, both in epidemiological and clinical studies.

RESUMO

Detecção quantitativa de oocistos de *Toxoplasma gondii,* por um teste modificado de Kato Katz usando coloração de Kinyoun (KKK), em gatos infectados experimentalmente com a cepa ME49

Detectamos oocistos de *Toxoplasma gondii* em fezes de gatos experimentalmente infectados, usando a abordagem de Kato Katz, com subseqüente coloração pelo método de Kinyoun. Animais sorologicamente negativos ao *T. gondii* foram infectados por via oral com $5x10^2$ cistos da cepa ME49 de cérebros de camundongos. Fezes foram colhidas diariamente a partir do 3º até o 30º dia pós-infecção. Oocistos foram detectados por centrífugo-flutuação em sacarose qualitativa e pelo método quantitativo de Kato Katz modificado corado pela técnica de Kinyoun (KKK). Em gatos experimentalmente infectados, oocistos foram detectados do 7º ao 15º dia pela técnica de centrífugo-flutuação em sacarose, mas oocistos foram detectados do 6º ao 16º dia pelo KKK, sendo sensível por um período maior, com documentação permanente. O pico da excreção de oocistos ocorreu entre 8º a 11º dia pós-infecção, antes de resultado sorológico positivo. KKK pode ser utilizado na triagem e quantificação da excreção de oocistos em fezes de animais suspeitos, com redução da manipulação de material infectante, diminuindo a possibilidade de contaminação ambiental e do operador.

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