J. Protozool. Res., 4, 48-55 (1994)

Characterization and Check of Identity of Different Strains of *Toxoplasma gondii* by Enzymatic Profiling

ANDREAS HASSL[1], MOHAMMED MANAFI[2], REGINA SOMMER[2], M. KUNDI[3], AND HORST ASPÖCK[1]

[1]*Department of Medical Parasitology,* [2]*Department of Water and Food Hygiene of the Clinical Institute of Hygiene, and* [3]*Institute of Environmental Hygiene, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria*

Received 29 November 1993 / Accepted 8 April 1994

Key words: *Toxoplasma gondii*, strain differentiation, enzyme profile, zymogram analysis, API enzyme research kit ®

ABSTRACT

Zymogram analysis of nine strains of *Toxoplasma gondii* all highly virulent for mice was performed with the API enzyme research kits®. The parasite strains had been maintained by continuous intraperitoneal infection in mice for many years separately in different laboratories in Europe and were retraced to five isolates (RH, BK, 928, KB, Alt). The API research kits® use chromogenic substrates for a semiquantitative detection of 84 enzymes, including the classes aminopeptidases, glycosidases, esterases, lipases, phosphatases and phosphoamidases. From the zymograms Manhattan distances were calculated and a hierarchical tree of the parasite strains was established by complete linkage.

All strains could be easily integrated into two distinct clusters. However, the correct designation of some strains as direct descendant of a definite isolate seems to be questionable. Zymogram analysis is suitable for a quick enzymatic characterisation of any mouse virulent Toxoplasma strain and for a simple comparison to defined reference strains. Thus, it allows a rapid detection of any strain confusion during isolation procedures or long term propagation.

INTRODUCTION

Toxoplasma gondii is an obligate parasite of a wide spectrum of mammals and birds with an intracellular growth and multiplication, but the trophozoites are viable and metabolically active outside host cells for some hours. The various strains of this parasite propagated throughout laboratories show different biological properties, considering virulence in mice or temperature-sensitivity as shown by Suzuki et al. (1989) and Waldeland et al. (1983). All these strains are morphologically indistinguishable. Thus, some simple ways of characterisation and differentiation of strains or isolates have to be developed. Moreover, very few comparative studies on biochemical characterisation of *Toxoplasma gondii* strains have been carried out, all of them are based on analysing isoenzyme patterns (Barnert 1990, Barnert et al. 1988, Darde et al. 1988, 1990, 1992). Although the enzymatic equipments of the Toxoplasma strains seem to differ considerably as demonstrated by Darde et al. (1988, 1992) and Manafi et al. (1993), several investigators (Darde et al. 1990, Sibley and Boothroyd 1992, Harmer et al. 1993, Manafi et al. 1993) have not been able to establish any correlation between genotype, enzyme zymodemes and hosts. Moreover, the methods having been

applied are not suitable for a rapid characterisation of strains of microorganisms or a check of identity.
During the last years standardized enzymatic procedures have been developed for the rapid identification of microorganisms. The API systems® (API Systems SA, Montalieu-Vercieu, France), which are the most frequently used miniaturized test kits for enumeration of bacteria (Manafi et al. 1991, Manafi and Rotter 1992), have been adapted by Manafi et al. (1993) for analyses of enzymes of *Toxoplasma gondii*. Using the API system® we have compared nine laboratory adapted Toxoplasma strains originally deriving from five isolates from different locations in Europe. The goal of this study was to analyse their degree of dissimilarity and to check the identity of different strains of the parasite, especially the ones derived from one isolate.

MATERIALS AND METHODS

The following strains of *Toxoplasma gondii* were examined: BK-V (BK-Vienna), RH-V (RH-Vienna), T, KB, 928, Alt, BK from Bonn (BK-B), BK from Magdeburg (BK-M), and RH from Magdeburg (RH-M) (sources and a brief characterisation of the strains are drawn up in Table 1).

Table 1:

strain [a]	grade of virulence [b]	source	year of isolation	place of isolation	cycle in h	reference
RH-V	+++	human brain	1939	USA	43	Sabin (1941)
BK-V	++	human CSF	1948	NL	48	Binkhorst (1948)
T	++	unknown	< 1976	?	43	Thalhammer pers.comm.
KB	+	cat heart	1966	NL	68	Overdulve (1978)
Alt	++	human fetus	1967	G	48	Werner (1967)
928	++	pig lung	1961	NL	43	Overdulve pers.comm.
BK-B	independently propagated at least since 1957 in Bonn; source unknown					Seitz pers.comm.
BK-M	independently propagated since 1982 in Magdeburg; source: Bonn					Müller pers.comm.
RH-M	independently propagated since about 1965 in Magdeburg; source unknown					Müller pers.comm.

[a] The strains had been obtained from the University Children's Hospital; University of Vienna; Austria (T); Institute of Medical Parasitology; University Bonn; Germany (BK-B); Institute of Medical Microbiology and Epidemiology; Medical Academy Magdeburg; Germany (BK-M, RH-M); State University of Utrecht; Netherlands (KB, 928).

[b] The grade of virulence was defined by determination of the 100% lethal dose (LD_{100}) of the strain inoculated intraperioneally in outbred Swiss mice. +++: $LD_{100} < 10$; ++ and +: $LD_{100} > 10$ and < 1000.

The strains had been obtained from different laboratories in Europe from 1974 - 1989 and, with the exception of RH-V and BK-V, had been stored in liquid nitrogen (-192°C). Before examination in the API system® all strains were maintained by serial intraperitoneal passage in outbred mice (female; 20 g; Him: OF1 (Swiss) SPF; source: Versuchstierzucht Himberg, Austria). For reducing the risk of analysing inhomogeneous populations each strain was continuously maintained for at least three months, each time passaging the peritoneal exsudate of one single mouse further. Right after harvesting on day 2 p.i. or day 3 p.i., respectively, the parasites, which are located almost exclusively extracellular at this moment, were washed three times in 145 mM sterile NaCl (10 min, 3000g), checked for purity (host cells/tachyzoites < 0.001, no bacterial contamination), counted and checked for integrity after staining a small portion with Diff-quick. Thereafter, the tachyzoites were suspended in sterile 10 mM phosphate buffer pH 7.5 to a density of $1X10^6$ cells/ml precisely.
Macrophages raised in mouse peritoneal cavities were treated the same way and used as a negative control. The results of a comparison between Toxoplasma trophozoites and host

cells enzyme equipments and the consequences for any control of purity are described in detail by Harmer et al. (1993).
The commercially available API ZYM® and the experimental API galleries aminopeptidases AP1-AP6, API osidases, and API esterases were used for enzyme profiling. The enzyme kits are semiquantitative micromethods using 84 chromogenic enzyme substrates, including 62 for aminopeptidases, 8 for glycosidases, 10 for esterases, and one for alkaline phosphatase, acid phosphatase, γ-glutamyltransferase, and phosphoamidase each. For API ZYM® only the results of valine arylamidase, leucine arylamidase, cystine arylamidase, trypsine, chymotrypsine, alkaline and acid phosphatases, and phosphoamidase were reported.
The test strips were placed in their moistened plastic incubation trays and 100 µl of the trophozoite suspensions were added to each cupule. All galleries were incubated for 13 h at 37°C. Enzyme reaction development was performed according to the manufacturer's recommendations and to the description of Manafi et al. (1993). The principle of these tests is based upon the enzymatic liberation of ß-naphtol and ß-naphtylamine groups from aryl-substituted substrates. The chromogen released is detected by coupling with a diazotized indicator. All tests were repeated at least once. The test reading was performed by eye in a semiquantitative manner which is the most sensitive and reliable evaluation possible (four intensities of coloration: transparent: 0, orange: 1, violet: 2, purple: 3).
The data were statistically analysed by calculating the city block distances and, thereafter, by hierarchical clustering (84 cases and four variables) in a personal computer with the software "Complete Statistic System"® (Statsoft; USA). The amalgamation rule for the construction of a hierarchical tree was a complete linkage.

RESULTS AND DISCUSSION

The intensities of the enzymic reactions are listed in Table 2.

Table 2:

	strain								
enzyme	RH-V	T	KB	928	Alt	BK-V	BK-M	BK-B	RH-M
AMINOPEPTIDASES									
L-Tyrosine arylamidase	1	1	1	0	1	0	0	0	0
L-Pyrrolidone arylamidase	0	0	0	0	1	0	0	0	0
L-Phenylalanine arylamidase	2	1	1	2	1	2	1	1	2
L-Lysine arylamidase	1	1	1	2	1	3	1	1	2
L-Hyroxyproline arylamidase	0	0	0	0	0	0	0	0	0
L-Histidine arylamidase	1	1	0	1	1	1	1	1	2
Glycine arylamidase	0	0	0	0	1	0	0	1	2
L-Asparate arylamidase	0	0	0	0	0	0	0	0	0
L-Arginine arylamidase	2	1	1	2	1	1	2	1	2
L-Alanine arylamidase	2	1	1	2	2	1	2	2	3
N-Benzoyl-leucine arylamidase	0	0	0	0	0	0	0	0	0
S-Benzyl-cysteine arylamidase	0	0	0	0	0	1	1	0	1
Methionine arylamidase	1	1	1	1	1	1	2	1	2
Glycyl-glycine arylamidase	1	1	1	1	1	1	2	1	1
Glycyl-phenylalanine arylamidase	1	1	1	1	1	1	2	1	1
Glycyl-proline arylamidase	1	3	3	3	2	3	3	2	1
Leucyl-glycine arylamidase	0	1	0	0	0	1	1	1	1
L-Seryl-tyrosine arylamidase	1	1	0	1	0	1	1	1	1
N-CBZ-arginine-4-methoxyarylamidase	0	0	0	0	0	0	0	0	0
L-Glutamine arylamidase	1	0	0	0	0	0	1	1	0
α-L-Glutamate arylamidase	0	0	0	0	0	0	0	0	0
L-Isoleucine arylamidase	0	0	0	0	0	0	0	0	0
L-Ornithine arylamidase	1	1	2	1	1	0	1	1	1
L-Proline arylamidase	0	0	0	0	0	0	1	0	0

L-Serine arylamidase	1	0	0	0	0	0	0	1	0
L-Threonine arylamidase	0	0	0	0	0	0	0	0	0
L-Tryptophane arylamidase	1	0	0	1	0	0	1	1	1
N-CBZ-glycyl-glycyl-arginine arylamidase	0	0	0	0	0	0	0	0	1
β-Alanine arylamidase	2	2	1	2	2	2	2	2	2
L-Alanyl-L-arginine arylamidase	1	2	1	2	1	2	2	1	2
L-Alanyl-L-phenylalanyl-L-proline arylamidase	1	2	2	2	2	2	2	2	2
L-Alanyl-L-phenylalanyl-L-prolyl-L-alanine arylamidase	0	1	0	1	2	1	2	1	2
L-Arginyl-L-arginine arylamidase	0	1	1	1	1	2	1	0	2
α-L-Aspartyl-L-alanine arylamidase	0	1	1	1	3	2	2	1	2
α-L-Aspartyl-L-arginine arylamidase	0	0	1	1	1	0	1	1	2
α-L-Glutamyl-α-L-glutamic arylamidase	0	0	0	0	0	0	0	0	0
α-L-Glutamyl-L-histidine arylamidase	1	1	0	0	0	1	1	1	2
Glycyl-L-alanine arylamidase	2	1	0	1	1	2	2	1	2
Glycyl-L-arginine arylamidase	1	1	0	1	0	2	1	0	1
Glycyl-L-tryptophane arylamidase	1	1	0	1	0	1	1	0	1
L-Histidyl-L-leucyl-L-histidine arylamidase	1	0	0	0	0	1	0	0	0
L-Histidyl-L-serine arylamidase	1	0	0	0	0	1	0	0	0
L-Leucyl-L-alanine arylamidase	2	2	0	0	1	1	1	1	1
L-Leucyl-L-leucyl-L-valyl-L-tyronyl-L-serine arylamidase	0	0	0	0	0	1	0	0	0
L-Lysyl-L-alanine arylamidase	0	0	0	1	0	1	1	0	1
L-Lysyl-L-lysine arylamidase	0	0	0	1	0	1	1	0	1
L-Phenylalanyl-L-arginine arylamidase	0	1	1	1	1	1	1	1	1
L-Phenylalanyl-L-proline arylamidase	2	2	3	2	3	1	2	3	3
L-Phenylalanyl-L-prolyl-L-alanine arylamidase	2	1	1	2	1	2	2	2	1
L-Prolyl-L-arginine arylamidase	1	0	0	1	0	1	1	0	1
L-Seryl-L-methionine arylamidase	1	1	1	1	1	1	2	1	2
L-Valyl-L-tyrosyl-L-serine arylamidase	0	0	0	0	0	0	0	0	0
N-Benzyl-L-alanine-4-methoxy arylamidase	0	0	0	0	0	0	0	0	0
N-CBZ-arginyl-4-methoxy arylamidase	0	0	0	0	0	0	0	0	0
N-Acetyl-glycyl-L-lysine arylamidase	0	0	0	0	0	0	0	0	0
L-Histidyl-L-phenylalanine arylamidase	0	0	0	0	0	2	0	0	2
L-Lysyl-L-serine-4-methoxy arylamidase	0	0	0	0	0	0	0	0	0
Leucine arylamidase	2	1	1	2	1	2	2	1	2
Valine arylamidase	0	0	0	0	0	1	1	0	1
Cystine arylamidase	0	0	0	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0
ESTERASES									
Esterases-C4	2	2	2	2	2	2	2	2	2
Esterases-C5	2	2	2	2	2	2	2	2	2
Esterases-C6	2	2	2	2	2	2	2	2	2
Esterases-C8	2	2	2	2	2	2	2	2	2
Esterases-C9	2	2	2	2	2	2	2	2	2
Esterases-C10	1	1	1	2	1	1	1	1	1
Esterases-C12	1	1	1	1	1	1	1	1	1
Esterases-C14	0	0	0	0	0	0	0	0	0
Esterases-C16	0	0	0	0	0	0	0	0	0
Esterases-C18	0	0	0	0	0	0	0	0	0
API ZYM									
Alkaline phosphatase	2	1	1	2	2	2	2	2	2
Acid phosphatase	2	2	2	2	3	3	2	2	2
Phosphoamidase	2	1	0	1	0	1	1	1	0
γ-Glutamyltransferase	0	0	0	0	0	0	0	0	0
GLYCOSIDASES									
α-Galactosidase	0	0	0	0	0	0	0	0	0
β-Galactosidase	1	0	0	0	0	0	0	0	0
β-Glucuronidase	1	0	2	0	0	0	1	0	0

α-Glucosidase	0	0	0	0	0	0	0	0	0
β-Glucosidase	0	0	0	0	0	0	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	0
α-Mannosidase	0	0	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0	0	0

Out of 84 enzyme reactions 50 (59,5%) were equally either positive or negative in all investigated strains. 24 enzymes were present in the trophozoites of all strains: 15 arylamidases, 7 esterases, alkaline and acid phosphatases. 26 enzymes were not detected in any of the samples. 52 enzymic reactions quantitatively differ within the nine strains. The city block distances calculated from these data are shown in Tab. 3. The hierarchical tree constructed by amalgamation is displayed in Fig. 1.

Manafi et al. (1993) have shown that the method of enzymatic profiling by API-systems® can be successfully adapted to an enzymatic characterization of *Toxoplasma gondii*. Thereby, several peculiarities in the enzyme patterns of this parasite were detected. The present study was devoted to the question of identity or degree of relationship of various strains of *Toxoplasma gondii* kept in different European laboratories. The API-systems® are miniaturised test kits for a quick demonstration of any activity of a large number of metabolic enzymes within a relatively small number of viable cells and for a rough estimation of the quantity of the activity. The non-standardised factors of this technique like e.g. the reading of results are compensated with the large number of enzymes of different enzyme families included into the study. Moreover, systematic errors within the application of these technique are of no relevance as long as similarities of cloned microorganism strains are investigated.

We compared nine strains of *Toxoplasma gondii* all of which are highly virulent for mice and all of which have been cultivated by serial intraperitoneal inoculation in mice. Our study had to be restricted exclusively to virulent and intraperitoneally maintained strains, as strains that are avirulent in mice can be produced in high amounts in tissue culture only. Yet, there are some hints that isoenzyme patterns may depend on the growth system (mice/tissue culture) at least to a certain degree (Darde et al. 1990, Hamer et al. 1993). To exclude a contamination of the trophozoite suspension with host cells or bacteria, control experiments with peritoneal cells as well as pure buffer were done simultaneously, impure suspensions were excluded from the analysis. Another point of flaws may result from differences of the rate of enzyme degradation after liberation of Toxoplasma trophozoites from host cells. Thus, careful attention had to be given to the timing of harvesting, and the optimal cycle time for a maximum yield of liberated trophozoites was determined as demonstrated in Tabl. 1. After harvesting, further processing was done as quick as possible, following the standard procedure for bacterial enumeration by API-systems®. Moreover, this proceeding eliminates the risk of introducing bacteria into the test system. Any bacterial contamination was prevented by the use of sterile buffers only.

Table 3:

	City-block (Manhattan) distances							
strain	RH-V	T	KB	928	Alt	BK-V	BK-M	BK-B
RH-M	39	48	48	30	35	26	26	21
BK-B	40	43	41	31	28	23	21	
BK-M	37	40	40	26	33	24		
BK-V	35	38	34	28	21			
Alt	34	31	35	29				
928	33	26	34					
KB	35	28						
T	29							

The methode applied in this study is designed the detect the quantity of activity of a large number of different metabolic enzymes of any microorganism strain, but it is not designed to attribute enzyme activity qualitatively to a set of isoenzymes. Nevertheless, genealogical conclusions drawn from the applications of the enzymatic profiling and of isoenzyme studies must show some correspondence to proove validity. From the nine strains examined in our system six had already been studied by a comparison of the isoenzyme patterns. This previous study by Barnert et al. (1989) has led to a hierarchical tree reflecting the possible phylogeny. Some of the basic results obtained by analysing the isoenzymes were confirmed in our recent study; in particular, both studies have revealed two basic groups of Toxoplasma strains. Moreover, the larger amount of our data allowed an improved interpretation and thus led to some modifications in the hierarchical tree.

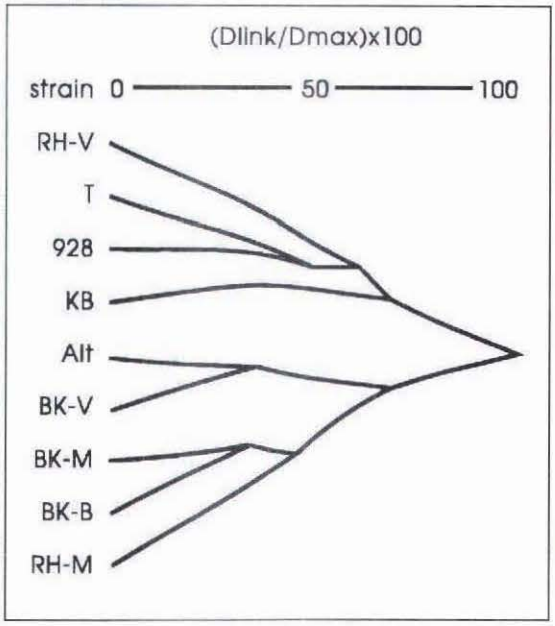

FIGURE 1. Hierarchical tree constructed from the city-block distances of the enzyme profile of *Toxoplasma gondii* with complete linkage as the amalgamation rule.

One result of our study is an elucidation of the relationship of the T strain. Due to its history of propagation, its attribution to either the RH or the BK cluster has always been suspected. According to our results the T strain is more similar to the Viennese RH strain than to any strain of the BK cluster. Another interesting result focuses on the position of KB strain. Barnert in 1990 had found a distinctly isolated position of this strain whereas we could now clearly classify the KB strain within the RH cluster.

Looking closer at the BK strains kept in various European laboratories, all studied BK strains have proved to be members of a single cluster (Fig. 1), a result confirming their common origin. Moreover, the BK strain from Magdeburg seems to be closer related to the BK strain from Bonn than to the Viennese BK strain. This result corresponds to the strains` history as known so far. The BK strain was transferred from Bonn to Magdeburg in 1982, whereas it has been propagated in Vienna for at least eight years longer. However, the strain Alt and - surprisingly - the strain RH from Magdeburg also fit in this BK-cluster. Due to these results one might postulate that some confusion during simultaneous propagation of BK and RH strains in Magdeburg and the Alt strain in Vienna may have occurred.

A study published by Sibley and Boothroyd (1992) demonstrated an essentially identical genotype in Toxoplasma strains virulent for mice isolated from different hosts on three continents. This supposed partial equality in the gen sequence does not necessarily contradict our results, as a semiquantitative enzyme determination depends on the level of enzyme expression and on enzyme degradation more than on any differences in the gen sequences. Until now, there are not any data available on the enzyme equipment of avirulent Toxoplasma strains. Zymodemes based on analyses of a few isoenzymes are not significant for a virulence determination (Darde et al. 1992).

Based upon a calculation of city block (Manhattan) distances a complete linkage was carried out in order to obtain a hierarchical tree demonstrating similarities between the strains (Fig. 1). We are of course aware of the fact that a complete linkage test does not necessarily lead to a tree which reflects the genealogy of the members included. Nevertheless, it may be ac-

cepted as a reasonable assumption that most of the similarities are to be traced back to evolutionary events and thus are of some phylogenetical significance. The tree in Fig. 1 demonstrates that the nine strains form two large groups, one of them consisting of the strains RH-V, T, 928, and KB; the other one comprises all BK strains, the strain Alt, and strain RH-M. If the distances between branches in fact reflect any genealogy has to remain unanswered. Yet, this technique provides a technical simple, inexpensive and quick way for checking the identidy of any mouse virulent Toxoplasma strain by comparison of its enzyme activity to the activities of defined reference strains.

ACKNOWLEDGEMENTS AND FOOTNOTE

The authors gratefully acknowledge the technical assistance of Ms. Silvia Blieweis, MTA, propagating the Toxoplasma strains. Moreover, we thank Dr. J.P. Overdulve, Utrecht, NL., Prof. Dr. H. Seitz, Bonn, G., Prof. Dr. K. Janitschke, Berlin, G., and Doz. Dr. W.A. Müller, Magdeburg, G. for kindly supplying us with Toxoplasma strains.
Parts of this paper were presented (by A.H.) at the first BIOMED Workshop on *Toxoplasma gondii* Research in Europe, Würzburg, 1993.

REFERENCES

Barnert, G. 1990. Charakterisierung verschiedener *Toxoplasma gondii*-Isolate mittels Isoenzym-Analysen. 132 pp. Thesis, University of Vienna, Vienna.

Barnert, G., Hassl, A., & Aspöck, H. 1988. Isoenzyme studies on *Toxoplasma gondii* isolates using Isoelectric Focusing. *Zbl. Bakt. Mikrobiol. und Hygiene, Series A 268:* 476-481.

Barnert, G., Hassl, A., & Aspöck, H. 1989. Isoenzym-Analysen zur Differenzierung von *Toxoplasma gondii*-Isolaten. *Mitt. Österr. Ges. Tropenmed. Parasitol. 11:* 19-24.

Binkhorst, C.D. 1948. Toxoplasmosis, a clinical, serological and histopathological study with special reference to the eye manifestations. 82 pp. Thesis, University of Leiden, Leiden.

Darde, M.L., Bouteille, B., & Pestre-Alexandre, M. 1988. Isoenzymic characteristization of seven strains of *Toxoplasma gondii* by isoelectrofocusing in polyacrylamid gels. *Am. J. Trop. Med. Hyg. 39:* 551-558.

Darde, M.L., Bouteille, B., & Pestre-Alexandre, M. 1990. Comparison of isoenzyme profile of *Toxoplasma gondii* tachyzoites produced under different culture conditions. *Parasitol. Res. 76:* 367-371.

Darde, M.L., Bouteille, B., & Pestre-Alexandre, M. 1992. Isoenzyme analyses of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. *J. Parasitol. 78:* 786-794.

Harmer, C., Hassl, A. & Aspöck, H. 1993. Vergleichende Untersuchungen über Enzymmuster unterschiedlich kultivierter Toxoplasmen. *Mitt. Österr. Ges. Tropmed. Parasitol. 15*: 113-118.

Manafi, M., Hassl, A., Sommer, R. & Aspöck, H. 1993. Enzymatic Profile of *Toxoplasma gondii. Let. Applied Microbiol. 16:* 66-68.

Manafi, M., Kneifel, W. & Bascomb, S. 1991. Fluorogenic and chromogenic substrates used in bacterial diagnostics. *Microbiol. Rev. 55:* 335-348.

Manafi, M. & Rotter, M.-L. 1992. Enzymatic profile of *Plesiomonas shigelloides. J. Microbiol. Methods 16:* 175-180.

Overdulve, J.P.1978. Excretion of *Toxoplasma gondii* by non-immunized and immunized cats: its role in the epidemiology of Toxoplasmosis. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen (Amsterdam), Series C 81:* 1-18.

Sabin, A.B. 1941. Toxoplasmic encephalitis in children. *J. Am. Med. Assoc. 116:* 801-807.

Sibley, L.D. & Boothroyd, J.C. 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature 359:* 82-85.

Suzuki, Y., Conley, F.K. & Remington, J.S.1989. Differences in virulence and development of encephalitis during chronic infection vary with the strain of *Toxoplasma gondii*. *J. Infec. Dis. 159:* 790-794.

Waldeland, H., Pfefferkorn, E.R. & Frenkel, J.K.1983. Temperature-sensitive mutants of *Toxoplasma gondii*: Pathogenicity and persistence in mice. *J. Parasitol. 69:* 171-175.

Werner, H. & Egger, I.1967. Vergleichende Untersuchungen an zystenbildende Toxoplasma-Stämmen. I. Mitteilungen: Über das Verhalten sog. schwach oder sog. avirulenter Toxoplasma-Stämme nach intraperitonealer und oraler Inokulation. *Zbl. Bakt. Mikrobiol. Hyg. A 206:* 259-266.