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Laboratory passage and characterization of an isolate of *Toxoplasma gondii* from an ocular patient in Korea

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Abstract: *Toxoplasma gondii* tachyzoites were isolated from the blood of an ocular patient, and have been successfully passaged in the laboratory, for over a year, by peritoneal inoculation in mice. The isolated parasite was designated the Korean Isolate-1 (KI-1) and its characteristics were compared with those of the RH strain, a well-known virulent strain originating from a child who suffered from encephalitis. The morphology, pathogenicity, infectivity and cell culture characteristics of the KI-1 were similar to those of the RH strain. Both RH and KI-1 antigens were detected by an anti-*T. gondii* monoclonal antibody (mAb), Tg563, against the major surface protein SAG1 (30 kDa), whereas no reaction was observed against an anti-*Neospora caninum* mAb, 12B4. The KI-1 was confirmed as an isolate of *T. gondii*. A long-term laboratory maintenance and characterization of a local *T. gondii* isolate is reported for the first time in the Republic of Korea.

Key words: *Toxoplasma gondii* Korean Isolate-1 (KI-1) and RH strain, ocular patient, immunoblotting, culture (in vitro), passage (in mice)

INTRODUCTION

Toxoplasma gondii is an intracellular protozoan that can cause significant morbidity and mortality in man and animals (Chai et al., 1997). In the Republic of Korea, the seroprevalence of *T. gondii* was reported to be around 2-7% among the general population in the 1980s (Choi WY et al., 1987), and about 7.7% in children younger than 10 years of age in the 1990s (Kook et al., 1999). Several clinical toxoplasmosis cases have been reported (Choi JS et al., 1980; Choi WY et al., 1997; Kim MH et al., 2000). The transmission of *T. gondii* occurs either by ingestion of oocysts shed in feline feces, cysts contained in chronically infected tissues of pigs or by vertical transmission from mothers to newborns. In most adult populations, *T. gondii* infection in general does not cause a serious illness, with the exception of immunocompromised patients. In congenital infections, however, this protozoan can cause abortion, blindness and mental retardation of the affected newborns.

For studies on various aspects of toxoplasmosis including diagnosis, treatment and immunization, local isolates of the parasites are needed. In patients

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infected with *T. gondii*, the parasites can spread into the whole body via the blood stream. Therefore, many sample types can be used to isolate *T. gondii*, including the bronchoalveolar lavage fluid (Gadea et al., 1995), amniotic fluid and placenta (Teutsch et al., 1980), saliva and tonsils (Amendoeira and Coutinho, 1982), blood (Hofflin and Remington, 1985) and cerebrospinal fluid (Fu et al., 1997). The infection can also be transmitted from the brain to the eyes via the optic nerve (Roberts and Mcleod, 1999).

In the Republic of Korea, the tachyzoites of *T. gondii* were isolated from the blood of two ocular patients (Choi et al., 1980). Unfortunately, however, neither of the two isolates was kept long enough for a long-term maintenance and characterization. We report here a successful isolation and maintenance of a Korean isolate of *T. gondii* from the blood of an ocular patient. Its virulence, tissue culture characteristics and immunoblot patterns were compared with those of the RH strain, a world-wide known virulent strain, which had originated from a child suffering from encephalitis (Høgdall et al., 2000). Our isolate was designated the Korean Isolate-1 (KI-1), and has been maintained in the laboratory, for over a year, by passages in mice.

MATERIALS AND METHODS

Brief history of the patient

A 61-year-old woman visited a local clinic with clinical complaints of visual disturbance and a retinal mass. The patient revealed a high intraocular pressure of 14 mmHg and low left eyesight of 0.2. Serological tests revealed typical patterns of an acute infection with increasing levels of IgM, 32.6 IU/ml (reference value; 0-6 IU/ml), and IgG, 5.14 IU/ml (reference value; 0-0.8 IU/ml), as measured by the enzyme immunoassay kits (Beckman Coulter, Inc., Chaska, Minnesota, U.S.A.). The antibody titer against *T. gondii* was 1 : 64 by the latex agglutination test (Eiken, Tokyo, Japan). The differential counts of WBC were as follows: neutrophils 27.4%, lymphocytes 58.1%, monocytes 11.7% and eosinophils 2.8%. After treatment with sulfadiazine and pyrimidine, the left eyesight of the patient increased to 0.3 and the intraocular pressure decreased to 12 mmHg. The lymphocytes and monocytes returned to their normal levels.

Isolation and maintenance of *T. gondii* (the KI-1)

The peripheral blood of the patient was drawn using a syringe, and transferred into a test tube. The blood was left to coagulate at room temperature, and the serum was taken out. The blood clot, with a small amount of serum, was frozen to -70°C, and thawed just before use. After thawing, the blood clot was minced with the tip of a pipette, and injected intraperitoneally into 3 young (6-week-old) female BALB/c mice. Seven days later, the mice were sacrificed and the exudates were drawn from the peritoneal cavity. The fluid was centrifuged at 2,500 rpm for 10 min at room temperature and the sediment was again inoculated intraperitoneally into 3 young mice. Several times of similar mouse passages were repeated. Each time when the peritoneal fluid was obtained, the sediment was smeared to detect *T. gondii* tachyzoites using a light microscope (LM) after Giemsa staining.

After identifying the presence of tachyzoites, the peritoneal fluid of the mice was inoculated into new young mice, and 3 days later the mice were sacrificed and the peritoneal fluids collected. Using this procedure the KI-1 isolate has been continuously maintained in our laboratory.

Transmission electron microscopy of the KI-1 tachyzoites

The KI-1 tachyzoites, obtained from the peritoneal fluid of BALB/c mice, were processed for transmission electron microscopy (TEM), following the procedure reported previously (Kook et al., 1995; Chai et al., 1997). Briefly, the tachyzoites were washed with cacodylate buffer (pH 7.4), and fixed with 2.5% glutaraldehyde at 4°C for 12 hrs. They were post-fixed with 1% osmium tetroxide for 2 hrs, and then dehydrated in a graded series of ethanol. They were embedded in epon, and semithin and ultrathin

sections were prepared. The ultrathin sections were stained with uranyl acetate and lead citrate, and observed using a TEM (1200 EX-II, JEOL, Japan) at 80 KV.

Infection of Sarcoma 180 cells with the KI-1

The Sarcoma 180 cell line was purchased from the Korea Cell Line Bank (Seoul, Korea). The cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NewYork, U.S.A.) supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were observed daily using an inverted LM.

Two culture flasks containing the Sarcoma 180 cells were each infected with 1×10^6 tachyzoites of the KI-1. A host cell-parasite ratio of 3 : 1 was used, as this ratio has been shown to yield a large number of intracellular parasites (Couatarmanach et al., 1991). The cell suspensions were collected from the flasks daily, by centrifugation at 1,500 rpm for 5 min from day 1 to day 6 post-infection (PI). The numbers of tachyzoites and cells in the decanted supernatant were counted in a hemocytometer and their viability determined by the trypan blue exclusion test. To avoid cell damages due to acidification of the medium, the RPMI 1640 medium was changed everyday.

Virulence test

Six-week-old, specific pathogen-free, BALB/c mice (females) were purchased from the Laboratory Animal Center in Seoul National University (Seoul, Korea), and intraperitoneally inoculated with variable doses (10^3, 10^4, 10^5, 10^6, 10^7 and 10^8) of *T. gondii* KI-1 and RH tachyzoites. The survival times of the mice were recorded from infection until day 8 PI.

Preparation of *T. gondii* and *Neospora caninum* antigens

The RH and KI-1 tachyzoites of *T. gondii*, harvested from the peritoneal exudates of experimentally infected mice, were purified using 40% Percoll (Pharmacia Biotech, Uppsala, Sweden) in phosphate buffered solution (PBS) (pH = 7.4) (Choi WY et al., 1992). The purified tachyzoites were disrupted by five cycles of freezing to -70°C and thawing at room temperature. The homogenate was centrifuged at 12,000 rpm at 4°C for 30 min and the supernatant used as the antigen.

The KBA2 isolate of *N. caninum* (Kim JH et al., 2000) was obtained from the Department of Pathology, Seoul National University College of Veterinary Sciences, and has been maintained in our laboratory by a previously described procedure (Kim JH et al., 2000). The procedure for preparation of the *N. caninum* antigen was the same as that for preparation of the *T. gondii* antigen.

Immunoblotting

The protein bands of the RH, KI-1 and *N. caninum* antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred onto polyvinylidene fluoride nitrocellulose membranes (Millipore Corporation, Bedford, Massachusetts, U.S.A.) (Towbin et al., 1979). The membranes were incubated at room temperature for 2 hrs with an anti-*T. gondii* monoclonal antibody (mAb), Tg563, at a 1:100 dilution (Catholic University of Korea, Seoul, Korea) and an anti-*N. caninum* mAb, 12B4, at a 1:100 dilution (Catholic University of Korea, Seoul, Korea). Horseradish peroxidase-conjugated goat anti-mouse serum (Cappel Company, St. Louis, Missouri, U.S.A.), at a 1:500 dilution, was used as the antibody. The blots were developed with 4-chloro-1-naphthol containing 0.03% H_2O_2 in phosphate buffer (0.01M, pH 7.4).

RESULTS

LM and TEM morphology of the KI-1 tachyzoites

Seven days after the second passage of the patient's blood clot into young BALB/c mice, the mice were sacrificed, and several drops of the peritoneal exudate were smeared on a glass slide and examined for *Toxoplasma* using a LM after Giemsa staining. *Toxoplasma* tachyzoite-like organisms were found in considerable numbers in 1 of the 3 mice. They were

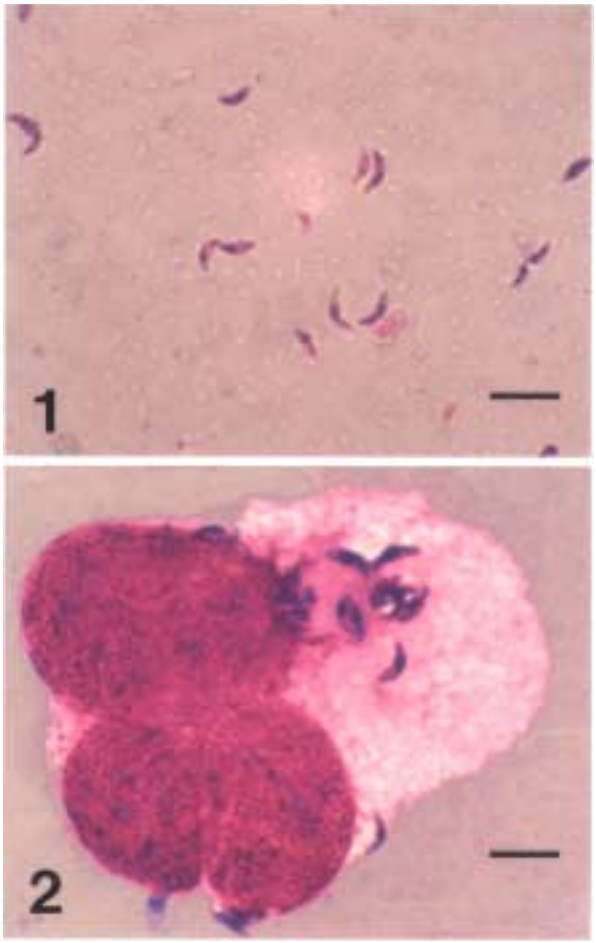

Fig. 1. The Korean Isolate-1 (KI-1) tachyzoites of *Toxoplasma gondii* isolated from an ocular patient. Giemsa stained. Scale bar = 12 μm. **Fig. 2.** The KI-1 tachyzoites of *T. gondii* in the Sarcoma 180 cell line cultured in vitro. Giemsa stained. Scale bar = 10 μm.

pyriform or crescent shaped (Figs. 1-2), measuring 4 to 6 μm long and 2 to 3 μm wide, each having an apparent nucleus. Their morphology was similar to that of the RH tachyzoites of *T. gondii*.

The TEM morphology of the KI-1 tachyzoites (Fig. 3) was consistent with that of the RH tachyzoites of *T. gondii* (Kook et al., 1995; Chai et al., 1997). The pellicle of the parasite was composed of two membranes forming sharp cell surfaces. The number of rhoptries was 6 to 8 in each tachyzoite. The nucleus had dense chromatins in the center as well as along the nuclear wall, and thus it was easily differentiated from the cytoplasm. Electron dense granules, ribosomes, vacuoles, and mitochondria were also seen (Fig. 3).

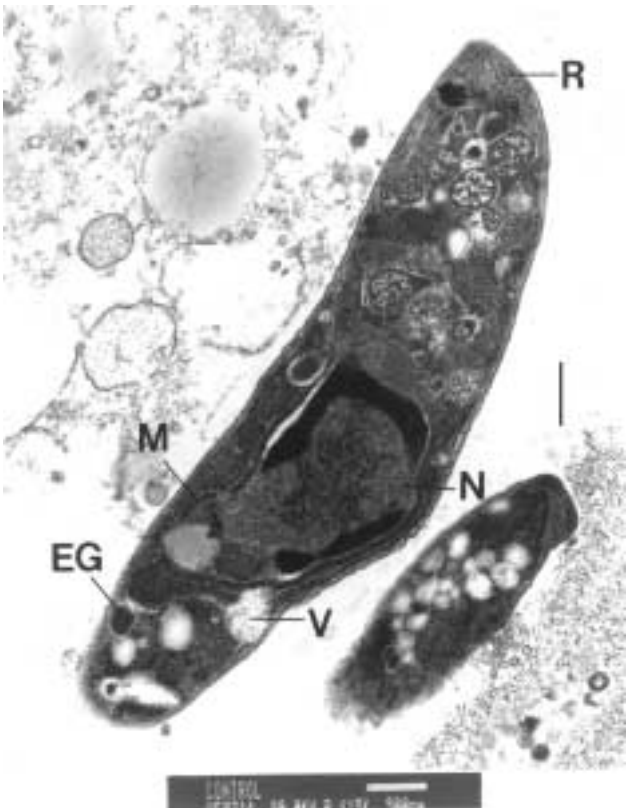

Fig. 3. TEM morphology of a KI-1 tachyzoite. The nucleus has dense chromatins in the center and along the nuclear wall, and are easily differentiated from the cytoplasm. Characteristic organelles such as the nucleus (N), rhoptries (R), electron dense granules (EG), vacuoles (V) and mitochondria (M) are seen. Scale bar = 0.5 μm.

Replication of tachyzoites in Sarcoma 180 cells

The Sarcoma 180 cells grew in suspension cultures as non-adherent polymorphic cells. The cell line was successfully infected by the KI-1 and intracellular multiplication of the parasites occurred. The KI-1 tachyzoites in the Sarcoma 180 cells (Fig. 2) presented a similar morphology to that observed in mice in vivo (Fig. 1). When 1×10^6 tachyzoites were seeded into the Sarcoma 180 cell suspension, containing 3×10^6 cells in RPMI medium, there was a 256-fold increase in the number of tachyzoites at day 5 PI; the number of tachyzoites and Sarcoma 180 cells being 2.56×10^8 and 1.23×10^5, respectively.

Table 1. Survival rates of mice infected with variable doses of *Toxoplasma gondii* RH or KI-1 tachyzoites

Inoculum dose of tachyzoites	*T. gondii* strain or isolate	Survival rates by day after tachyzoite inoculation (%)						
		2	3	4	5	6	7	8
10^8	RH	40[a)]	0	0	0	0	0	0
	KI-1	60	0	0	0	0	0	0
10^7	RH	100	60	0	0	0	0	0
	KI-1	100	60	0	0	0	0	0
10^6	RH	100	100	40	0	0	0	0
	KI-1	100	100	80	0	0	0	0
10^5	RH	100	100	100	40	0	0	0
	KI-1	100	100	100	60	20	0	0
10^4	RH	100	100	100	60	20	0	0
	KI-1	100	100	100	60	40	0	0
10^3	RH	100	100	100	100	40	40	0
	KI-1	100	100	100	60	40	40	0

[a)]Two of 5 inoculated mice survived (n = 5).

Virulence

The virulence of the KI-1 tachyzoites was estimated by observing the survival of the mice after intraperitoneal inoculation, and compared with that of the RH tachyzoites (Table 1). At the inoculum dose of 10^8 tachyzoites per mouse, only two of five (40.0%) and three of five (60.0%) mice, for the RH and KI-1 groups, respectively, survived to day 2 PI, and no mice survived longer than 3 days from either group (Table 1). At the dose of 10^7 tachyzoites per mouse, mice in both groups survived equally, with the survival rate of 60.0% at day 3 PI. At the dose of 10^6 tachyzoites per mouse, however, the survival rate on day 4 PI was different between the two groups, 40.0% and 80.0% for the RH and KI-1 groups, respectively. At the dose of 10^5 tachyzoites per mouse, the survival rate on day 5 PI was 40.0% and 60.0%, and that on day 6 PI was 0% and 20.0%, for the RH and KI-1 groups, respectively. The survival of mice was similar between the two groups at the inoculum doses of 10^4 and 10^3 tachyzoites per mouse. These results showed that the KI-1 is an isolate with strong virulence, comparable to that of the RH strain.

Immunoblot patterns

Immunoblotting of the RH and KI-1 antigens showed that both antigens reacted to the mAb (Tg 563) against the *T. gondii* SAG1 antigen (30 kDa), but

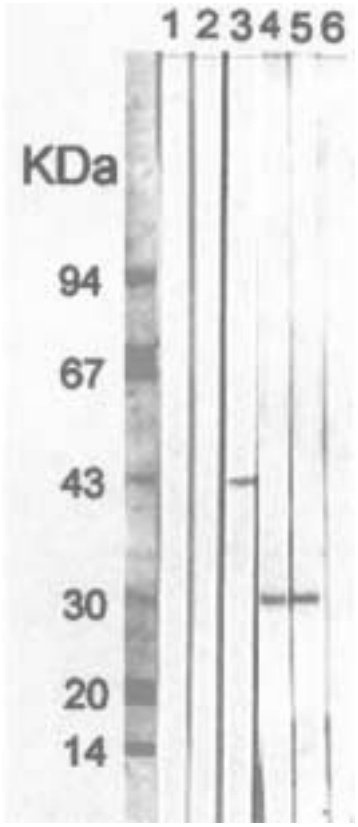

Fig. 4. Immunoblot of the RH and KI-1 of *T. gondii* and *Neospora caninum* KBA2 antigens, with the anti-*Toxoplasma* monoclonal antibody (mAb) Tg563 and anti-*Neospora* mAb 12B4. The RH, KI-1 and KBA2 antigens were reacted with the 12B4 (Lanes 1, 2 and 3), and the RH, KI-1 and KBA2 antigens were reacted with the Tg563 (Lanes 4, 5 and 6).

not to the mAb (12B4) against the *N. caninum* antigen (Fig. 4). The mAb, 12B4, reacted only to the *N. caninum* antigen (Fig. 4).

DISCUSSION

Relatively lower prevalences and antibody titers of *T. gondii* have been reported among Koreans compared with U.S. and Europian populations (Choi et al., 1992; Kook et al., 1999). This might be related to the food-eating habits (rarely consume raw or improperly cooked pork) and pet-loving fashions (prefer puppies to kittens) of the Korean people. In addition, clinicians who are working in the field of infectious diseases in the Republic of Korea have paid little attention to toxoplasmosis. Moreover, the sensitivity of serological tests may be low; *T. gondii* may be present in the saliva of patients in early stages of infection, when the infection cannot be detected by the serological tests (Amendoeira and Coutinho, 1982). Isolation of the *T. gondii* parasite is necessary in such patients, whether their serological tests are positive or negative.

In the Republic of Korea, the organism responsible for human toxoplasmosis was isolated from 4 patients, i.e., 2 congenital (Chung et al., 1980) and 2 chorioretinitis patients (Choi et al., 1980). Unfortunately, however, the properties of the isolates were not characterized, and the isolates were not maintained in the laboratory.

The present study describes, for the first time in the Republic of Korea, a successful isolation and characterization of *T. gondii* from a patient, with long-term (over a year) laboratory maintenance of the isolate (KI-1) by mouse inoculation. The morphology, antigenicity and virulence of the KI-1 tachyzoites were similar to those of the RH strain (Kook et al., 1995; Chai et al., 1997). The KI-1 survived and actively multiplied in the Sarcoma 180 cells; a 256-fold increase in the number of tachyzoites was observed at day 5 PI. Immunoblotting revealed that both the RH and KI-1 antigens reacted specifically to an anti-*T. gondii* mAb, but not to an anti-*N. caninum* mAb. Based on these results, the KI-1 was confirmed to be an isolate of *T. gondii*.

Since *T. gondii* was first described in a rodent, numerous strains, or isolates, have been obtained from humans. For example, *T. gondii* was isolated from the saliva and tonsils of a 3-year-old child and maintained by mouse inoculation (Amendoeira and Coutinho, 1982). Tachyzoites were obtained from the blood of an acquired immunodeficiency syndrome (AIDS) patient, and maintained by tissue cultures (Hofflin and Remington, 1985). Three isolates were obtained from the cerebrospinal fluid of humans and have been maintained by mouse inoculation at the Zhejiang Academy of Medical Sciences, China (Fu et al., 1997).

The strains of *T. gondii* were clustered into 2 major virulence groups, virulent (including the RH) and avirulent (including the ME49), with the virulent strains comprising of only a single clonal lineage (Sibley and Boothroyd, 1992). More recently, however, 3 clonal lineages were identified among *T. gondii* using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analysis; type I, II and III (Howe and Sibley, 1995). The types I and II were virulent (including the RH) and avirulent strains (including the PLK), respectively, and the type III consisted of avirulent and less virulent isolates (Howe and Sibley, 1995). However, evidence was shown that not all the virulent isolates are closely related. For example, the strain MAS, highly pathogenic to mice, was consistent with the type I in only 4 of 6 allelic loci studied; different patterns were observed in the remaining 2 loci, i.e., L328 and 62 (Howe and Sibley, 1995). The type I strains were most frequently seen in congenital toxoplasmosis of humans, whereas the type II predominated among AIDS patients; the type III was most common in animal infections (Howe and Sibley, 1995). It is interesting to note that the KI-1 is a strongly virulent isolate, and seems to belong to the type I. However, studies on the DNA patterns of the KI-1 are needed.

The sequence polymorphism has also been studied in a number of DNA loci of different *T. gondii* strains, including the genes for the heat-shock protein 70 (Lyons and Johnson, 1998), a dense granule antigen

GRA6 (Fazaeli et al., 2000a) and the intergenic spacer (Fazaeli et al., 2000b). The sequence variants of *T. gondii* for those genes were used as markers for distinguishing *T. gondii* isolates from different animals and geographical localities (Høgdall et al., 2000). Therefore, analyses of the sequence heterogeneity of the KI-1 genes should be performed. Research on the development of diagnostic kits and vaccines using the KI-1 is also needed.

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