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A *Toxoplasma gondii* strain isolated in Okinawa, Japan shows high virulence in Microminipigs

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ABSTRACT

Toxoplasma gondii strains have been isolated all over the world and their virulence has been examined mainly using laboratory mice. However, *T. gondii* differs in virulence depending on the host animal species. Therefore, to evaluate the virulence of each strain in domestic animals, it is necessary to examine using not only mice but also the concerned animals. We have shown that TgCatJpOk4, a *T. gondii* strain recently isolated in Okinawa, Japan, has a high virulence against laboratory mice, comparable to highest virulent RH strain in mice; however, the virulence to domestic animals remains unknown. In this study, we examined the virulence using the Microminipig. After infection, four out of five infected pigs showed severe clinical symptoms: inappetence, hypoactivity and tachypnea. Eventually, three out of the five infected pigs succumbed before the end of the observation. Among the three dead pigs, histological analysis revealed that interstitial pneumonia and spotty necrosis in the liver indicating that the TgCatJpOk4 strain has a high virulence not only in laboratory mice, but in pigs as well.

1. Introduction

Toxoplasma gondii is a highly prevalent protozoan parasite that infects all warm- blooded animals, including humans. Most *T. gondii* infections in humans are generally asymptomatic, but in immunocompromised individuals, fatal encephalitis, myocarditis and pneumonitis are reported [1]. In addition, infection of *T. gondii* during pregnancy can cause severe damage to the fetus and induce abortions and stillbirths [1]. The reproductive pathogenicity of *T. gondii* on ruminant animals has also been reported [2]. Although the majority of cases of *T. gondii* infection in pigs are asymptomatic, clinical toxoplasmosis sometimes occurs in young pigs [3]. Although in Japan, clinical toxoplasmosis in pigs has almost been eradicated [4], pig toxoplasmosis remains a severe problem in the global pig industry.

Extensive research has succeeded in isolating several strains of the *T. gondii,* and the virulence of these strains has been determined using laboratory mice [5]. For most strains isolated in North America and Europe, the virulence in mice has a strong correlation to the genotype [6]. With the exception of a few atypical strains, *T. gondii* isolated in North America and Europe are classified into only 3 lineages: Types I, II and III with high, low and no virulence in mice, respectively [6,7]. Type I parasites exhibit a 100% lethal dose (LD_{100}) profile in mice upon injection with 10^0 parasites. On the other hand, Type II has a lower virulence ($LD_{50} > 10^3$) and Type III is avirulent ($LD_{50} > 10^5$) [6,7]. However, it has recently been reported that Type I parasites exhibit virulence only in laboratory mice and that some mouse strains derived from wild mice show resistance to the *T. gondii* Type I strain [8,9]. The association between genotype and virulence of *T. gondii* in other host

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species is not yet clearly understood. Actually, we recently reported a Japanese isolate, TgCatJpGi1/TaJ, which shows high virulence in mice but no virulence in pigs [10]. This result suggests that *T. gondii* differs in virulence depending on the host animal. It is therefore impossible to estimate the virulence of each *T. gondii* strain in domestic animals, based on the virulence data obtained using laboratory mice.

A *T. gondii* strain, TgCatJpOk4, was recently isolated in Okinawa, Japan [11]. This strain is not classified as Type I, II or III, but is closely related to Type II by phylogenetic analysis using six genes of *T. gondii* (UPRT intron1, UPRT intron7, HP intron2, GRA6, GRA7, SAG1) [11]. Although Type II strains usually show low virulence in laboratory mice [6], this strain exhibits a high virulence in laboratory mice, comparable to the most virulent Type I strain. All mice injected with only 10 tachyzoites of TgCatJpOk4 strain died within 12 days [11]. However, the virulence in other animals without mice are unrevealed. In this study, using Microminipigs, we examined the virulence of a *T. gondii* strain, TgCatJpOk4. In this study, we used Microminipigs (Fuji Micra Inc., Shizuoka, Japan) as the experimental animal. The small body size, BW of young matured Microminipig (< 10 kg), is a great advantage as an experimental animal [12]. Microminipig is a brand name and registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine. Microminipig is often used as experimental animal models in the fields of pharmacology, toxicology and infectious diseases [13–15].

2. Materials and methods

2.1. Animals and parasites

Microminipigs (5 months old, females) were purchased from Fuji Micra Inc. (Fujinomiya, Japan). All the experiments using animals were performed in accordance with the Gifu University Animal Care and Use guidelines from the Committee (Permission No. 15078). Vero cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 7.5% fetal calf serum plus 20 μg/ml of gentamicin. TgCatJpOk4 [11] and PLK strains were maintained in Vero cell culture as tachyzoites.

2.2. Infection

To purify TgCatJpOk4 tachyzoites from the Vero cell cultures, the infected Vero cells were broken by passage through a 27 G needle three times and then centrifuged at 2000 rpm for 10 min. To remove the host cell debris, the pellet was suspended in 10 ml of RPMI medium and filtered with a five-micrometer filter. Parasite numbers were counted using a cell counter, and 1×10^7 tachyzoites were injected intraperitoneally into each Microminipig. After infection, symptoms and body temperature were observed and recorded periodically. Blood samples were also collected regularly.

2.3. Haematology and serum biochemistry

Blood samples (3–5 ml) with EDTA from all pigs were processed for automatic white blood cell and platelet concentration count (Celltaqα, NIHON KOHDEN, MEK-6358). The serum biochemistry was assayed in a semi-automatic spectrophotometer (DRI-CHEM 7000 V, FUJIFILM). The gray areas in Fig. 2 indicate the reference range of same-aged Microminipigs [16].

2.4. Histopathology and immunohistochemistry

At 22 days post infection, pigs were sacrificed by an overdose of thiamylal sodium (Isothol, 500 mg) (Nichi-Iko, Toyama, Japan). The lung, liver, brain, kidney, spleen, heart, small intestine and lymph nodes were collected from each pig and fixed in 5% formaldehyde solution. The organs of pigs that died before the end of the examination were collected immediately after their death and were subjected to paraffin embedding and histological processing. Subsequently, we performed microtomy to obtain 5 μm thick histological slices for each tissue, which were placed on glass slides, stained with hematoxylin-eosin (HE), and mounted with a cover glass. The sections of the lungs were subjected to immunohistochemical examination. This examination was performed using a ready-to-use rabbit polyclonal anti-*T. gondii* antibody (BioGenex; San Ramon, CA, B-AR1255R) and a mouse monoclonal anti-CD204 antibody (TransGenic Inc.; Fukuoka, Japan, clone SRA-E5, 1:800 dilution) as the primary antibody after autoclave pretreatment for antigen retrieval (121 °C for 15 min in Target Retrieval Solution (10×, pH 6.0) (DAKO; Hamburg, Germany, Code S1699)). EnVision™ + System Labelled Polymer-HRP anti-rabbit (DAKO; Hamburg, Germany, Code K4002) was used as the secondary reagent.

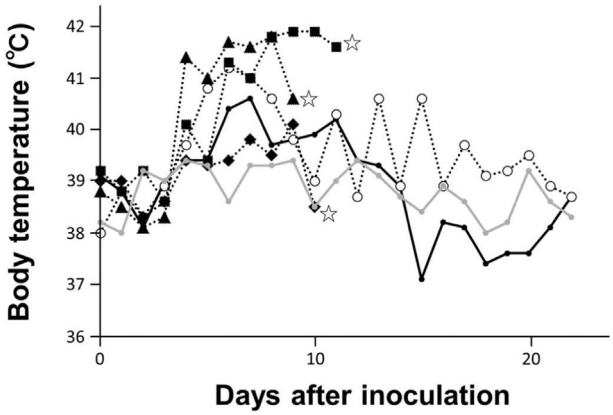

Fig. 1. Body temperatures of the experimental pigs.
Five pigs (pig a, b, c, d and e) were injected intraperitoneally with 10^7 tachyzoites. Pig f was not injected as a negative control. After the infection, the rectal temperature was measured periodically. The black and gray solid lines represent the data of pig a and pig f, respectively. The dotted lines indicate the data of pigs which showed clinical symptoms during the experiment: pig b (white circle), pig c (diamond), pig d (square) and pig e (triangle). Stars indicate the timing of death.

2.5. Western blotting

Crude tachyzoite antigen solution was prepared from PLK strain tachyzoites as previously described [17]. A 15-μl aliquot per well of the antigen was loaded on a 5–20% SDS gel. After electrophoresis, the antigens were transferred onto a nitrocellulose membrane using the Trans-Blot® SD Semi-dry Transfer Cell (Bio-Rad, Hercules, CA, USA) for 90 min followed by blocking in 3% skimmed milk overnight at 4 °C. The membrane was then incubated in Microminipig serum (diluted 1:1000 in 3% skimmed milk) at 37 °C for 1 h. The membrane was washed 5 times (5 min each) in PBS-Tween® 20 (PBS-T). The membrane was again incubated in a rabbit anti-pig IgG-HRP secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) (diluted 1:20000 in 3% skimmed milk) at 37 °C for 1 h, and finally washed in PBS-T 5 times (5 min per wash). Amersham ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, England, UK) was used to visualize the reactive bands, according to the manufacturer's instructions, and the image was acquired using ChemiDoc™ XRS + system (Bio-Rad Laboratories). The serum of an uninfected Microminipig was used as the negative control serum. The serum of a Microminipig which had been infected with 10^5 tachyzoites of TgCatJpGi1/TaJ/GRA Red strain [17] was collected at 119 days after infection and used as the positive control.

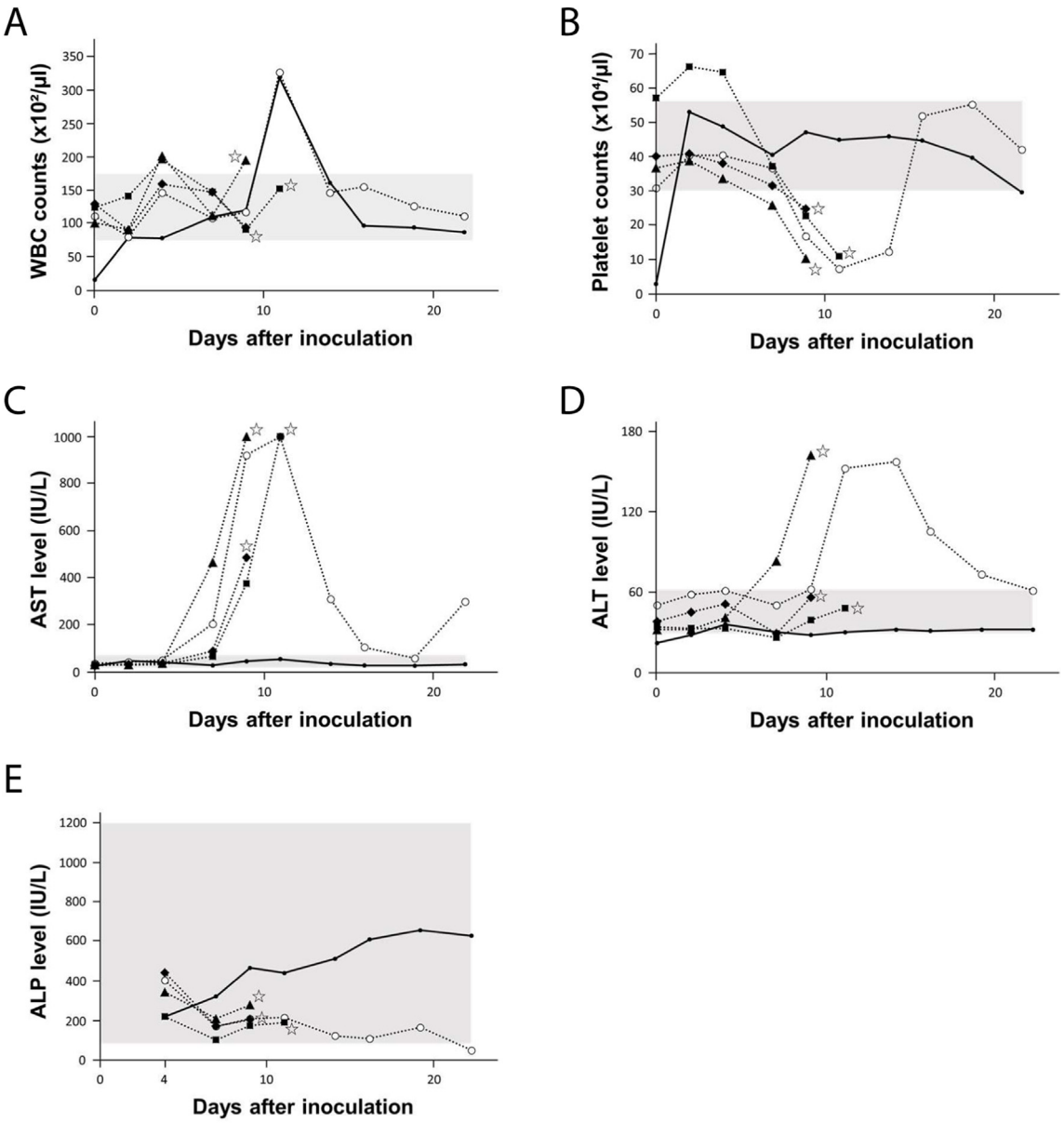

Fig. 2. The haematological and serum biochemistry parameters.
Each panel shows the results of WBC number (A), Platelets number (B), AST (C), ALT (D) and ALP (E) concentrations. The black solid line represents the data of pig a. The dotted lines indicate the data of pigs which showed clinical symptoms during the experiment: pig b (white circle), pig c (diamond), pig d (square) and pig e (triangle). Stars indicate the timing of death.

3. Results

3.1. Virulence of T. gondii TgCatJpOk4 strain in pig

Five Microminipigs (5 months old; identified with alphabets a-e) were intraperitoneally injected with 10^7 TgCatJpOk4 strain tachyzoites. The body temperatures of all infected pigs went over 40 °C at least once (Fig. 1). Four of the five infected pigs showed inappetence, fever, hypoactivity and tachypnea from the 4th–7th day after the infection (pigs b, c, d and e). With the exception of a temporary increase in temperature, one infected pig did not show any clinical symptoms during the entire period of the experiment (pig a). Among the four pigs that showed clinical symptoms, three pigs died during the observation (pigs c, d and e). The clinical symptoms, i.e. inappetence, fever, hypoactivity and tachypnea, of one pig (pig b) disappeared on the 12th day after the infection and the body temperature also returned to normal (Fig. 1).

3.2. Haematological and serum biochemistry parameters

Although the white blood cell (WBC) count of two of the pigs which died during the observation (pigs c and d) was within the reference range, the WBC count of the third pig which died (pig e) increased above the reference range at 9 days after injection, just before death (Fig. 2A). On the other hand, the WBC count of the two pigs that survived (pigs a and b) transiently increased above the reference range at 11 days after infection (Fig. 2A). For the pigs that showed clear clinical symptoms (pigs b, c, d and e), the number of platelets decreased below the reference range during the acute phase (Fig. 2B). This decrease in the platelet count was not observed in the pig which showed no symptoms except for a temporal fever (pig a) (Fig. 2B). The serum alanine aminotransferase (ALT) levels of the four pigs, which showed clear clinical symptoms, increased beyond the reference range (Fig. 2C). Serum aspartate aminotransferase (AST) levels also increased in the pig that recovered (pig b); however. by 14 days after the infection, the

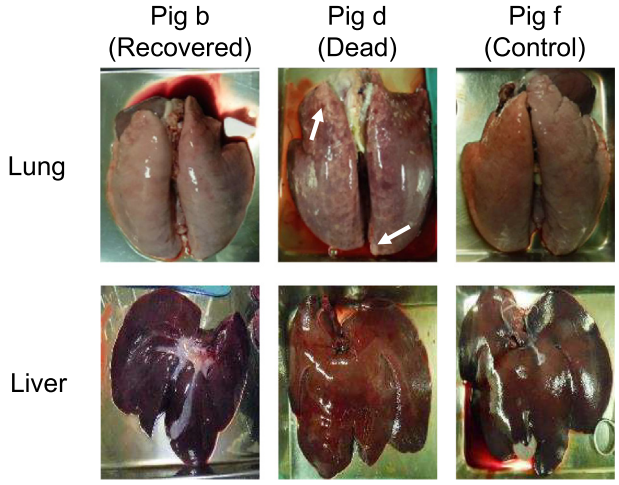

Fig. 3. The macroscopic alterations of organs of infected pigs.
Upper panels: The lung of a pig survived during observation (pig b), a pig died with acute toxoplasmosis (pig d) and a control pig (pig f). Arrows show whitish spots on the surface of the lung. Lower panels: The liver of pig b, pig d and a control pig (pig f).

increased AST levels had decreased to near-reference range levels (Fig. 2C). The serum ALT and AST levels of the infected pig which showed no clinical symptoms except a temporal fever (pig a) remained within the reference range throughout the observation (Fig. 2C and D). In addition, the serum alkaline phosphatase (ALP) levels of all five infected pigs remained within the reference range during the observation (Fig. 2E).

3.3. Lesion morphology

On the 22nd day after the infection, pigs that survived were sacrificed, and the lung, liver, brain, kidney, spleen, heart, small intestine and lymph nodes were obtained for the autopsy. The organs of pigs which died during the experiments were obtained and fixed just after their death for further examinations. Regardless of the increase in ALT and AST (Fig. 2C and D), no obvious abnormalities were found in the macroscopic examination of the liver of all infected and non-infected pigs (Fig. 3). In contrast, microscopic analyses revealed many spotty necrosis and leukocytes infiltration in the portal region in the liver of the infected pigs (Fig. 4A). The macroscopic appearance of the brain, kidney, spleen, heart, small intestines and lymph nodes were also similar between infected pigs and the non-infected control pig (data not shown). In contrast, the lungs of all three pigs which died during the observation (pig c, d and e) were diffusely reddened and had several

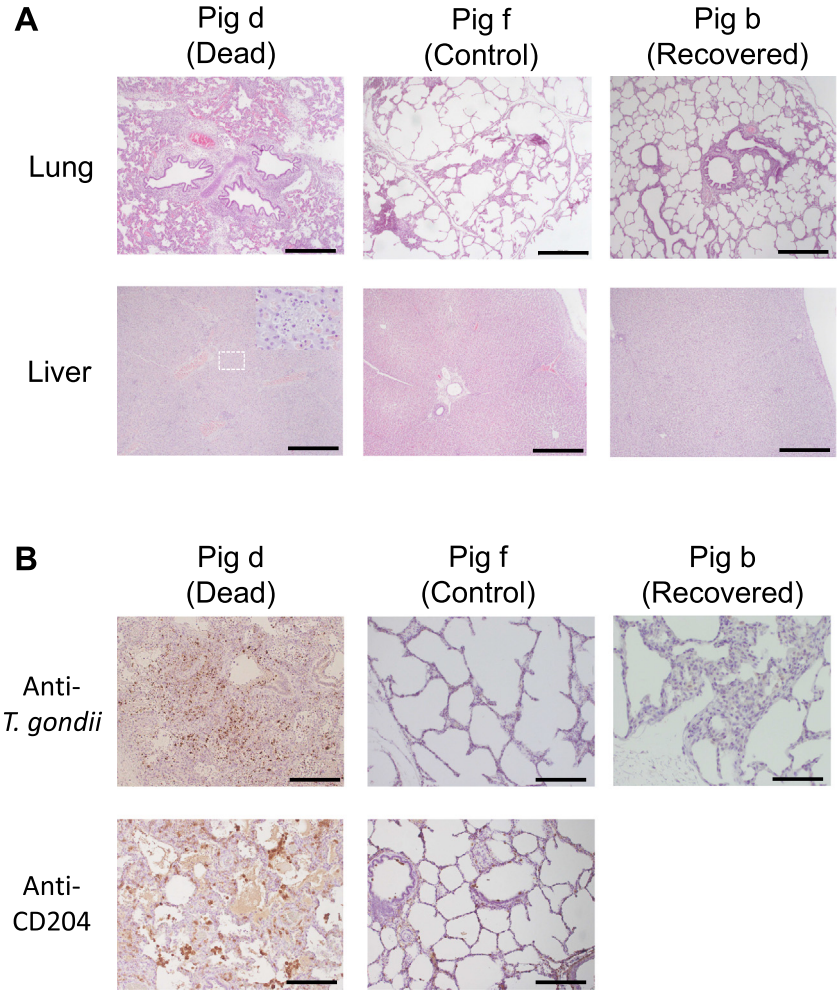

Fig. 4. Histological features of the experimental pigs.
A. The HE stained tissue sections of the lung and liver of pig b, pig d and a control pig (pig f). Scale bar = 500 μm. Inserted image (pig d liver) is an enlarged figure of areas indicated by the dotted line, a necrosis focus. B. Immuno-stained lung tissue sections of pig b, pig d and a control pig (pig f) with anti- *T. gondii* antibody, and pig d and a control pig (pig f) with anti-CD204 antibody. Scale bar = 200 μm.

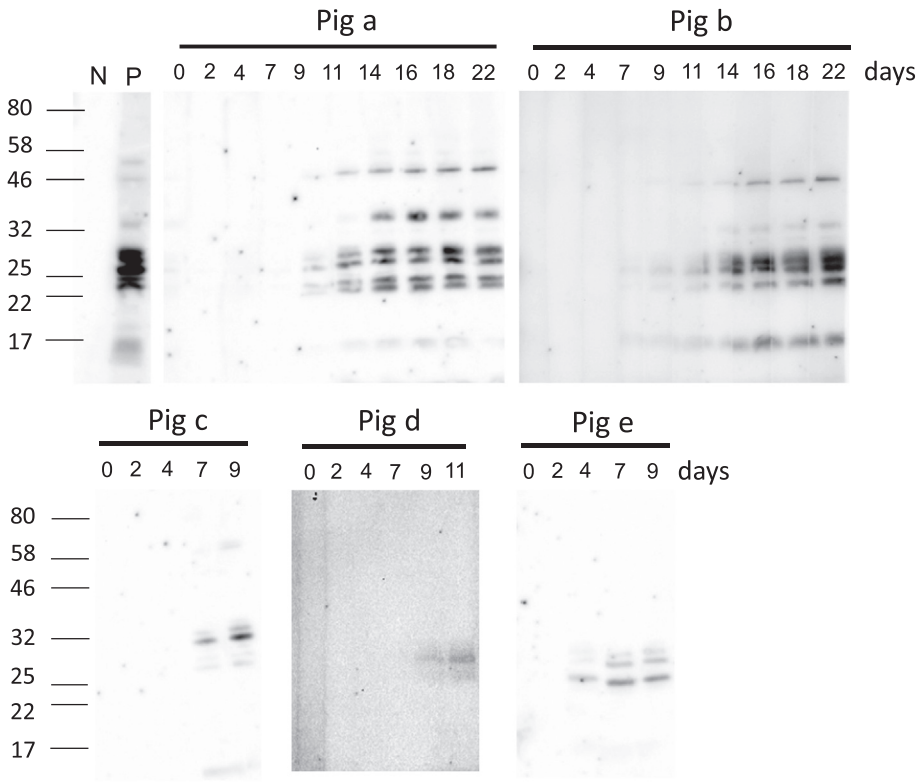

Fig. 5. Production of anti-*T. gondii* Antibodies.
Serum samples collected over time from the five infected pigs were assayed by Western blotting against tachyzoite lysate. The number above each lane indicates the day post infection. N: negative control using serum from an uninfected Microminipig. P: positive control using serum of a Microminipig infected with 10^5 tachyzoites of TgCatJpGi1/TaJ/GRA Red strain, 119 days post infection. [17]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whitish spots (Fig. 3). Microscopic analyses also revealed that numerous leukocytes infiltration in the bronchial septa and the alveolar septa, and those septa were severely thickened (Fig. 4A), representing the pathological characteristics of interstitial pneumonia. The recovered pig after a temporal fever showed mild leukocyte infiltration at around the bronchioles (pig b). For further analysis of inflammation in the lungs of the dead pigs, we performed immunohistochemical analysis. A part of the inflamed leukocytes was positive for CD204, a monocytes/macrophages marker (Fig. 4B). Moreover, the presence of a large number of parasites in the lung of dead pigs was confirmed by immunolabeling with anti-*T. gondii* antibody (Fig. 4B). No parasites were detected in the lung of the pig that recovered (pig b) (Fig. 4B). We could not detect any significant pathological changes among brain, kidney, spleen, heart, small intestines and lymph node by the H&E staining between infected pigs and the non-infected control pig (data not shown).

3.4. Production of anti-T. gondii antibodies

To confirm the production of anti-*T. gondii* antibodies, infected pig sera were assayed using tachyzoite lysate by Western blotting. The three pigs which showed clear clinical symptoms and died during the acute phase (pigs c, d and e) produced antibodies which reacted to an approximately 25–30 kDa antigen (Fig. 5). Other infected pigs which survived during the observation (pigs a and b) also produced antibodies reacting with several tachyzoite antigens including the approximately 25–30 kDa antigen (Fig. 5).

4. Discussion

This study revealed that TgCatJpOk4 strain exhibits a high virulence in Microminipigs. The observation in the lungs was consistent with previous findings of acute toxoplasmosis in pigs caused by highly virulent *T. gondii* strains [18]. In the past studies, it was reported that cloudy swellings and scattered whitish spotty lesions were observed in the liver of pigs that died of toxoplasmosis [19]. However, in our experiment, regardless of the high ALT and AST levels, no lesions were observed macroscopically in the liver of infected pigs. There is a possibility that the period from infection to death was too short for lesion formation in the liver. Considering the severe tissue damage and inflammation in the lungs, coupled with the lack of morphological abnormalities in other organs, the cause of death of the three pigs was possibly as a result of pneumonia. All infected pigs, except the one pig without clinical symptoms (pig a), showed platelet counts below the reference range. It is known that a decrease in platelets is accompanied by disseminated intravascular coagulation (DIC) caused by the inflammatory response to infectious diseases [20]. Although there were no observations which suggest DIC in our experiment, it has been reported that purpura, one of the clinical signs of DIC, is a common clinical sign in the case of pig toxoplasmosis [21]. One pig (pig a) did not show any symptoms after the infection, except for a slight and temporal fever. However, a transient increase in the WBC number, as well as the production of antibodies against *T. gondii* were observed in this pig. Although we could not find parasites in the tissues of this pig (data not shown), these results strongly suggest that *T. gondii* infection was established in this pig.

Compared to previous reports studying the virulence of *T. gondii* strains in pigs, the virulence of TgCatJpOk4 is considered to be extremely high. Miranda et al. [22] reported that after injecting 1×10^7 tachyzoites of the Type I RH strain, which is one of the most virulent strains in laboratory mice, into 8-month-old BR-1 mini pig, acute symptoms were observed, but all the infected pigs recovered and survived [22]. The infection of the Type II ME49 strain did not kill any pigs either [22]. In contrast, three out of five pigs died after infection with TgCatJpOk4 strain. TgCatJpOk4 strain might have a higher virulence in pigs than the RH strain, which is the most virulent strain in mice. In Japan, clinical toxoplasmosis in pigs has been reported only from the Okinawa prefecture, but no case has been reported from the other 46 prefectures since 2010 [4]. However, seropositive pigs have been identified all over Japan [23]. The reason for the limitation of pig toxoplasmosis to only Okinawa prefecture, despite widespread seropositivity in pigs in other prefectures, is not yet known. It might be

because the distribution of *T. gondii* strains with high virulence in pigs, such as TgCatJpOk4, is limited to Okinawa.

Differences in anti-parasitic responses in mice and humans have been previously reported. Interferon-γ related GTPase and inducible nitric oxide synthase (iNOS) are essential for parasite resistance in mice [24]. In contrast, interferon-γ related GTPase and iNOS do not protect human cells from *T. gondii* infection [25]. Rather, in human cell lines, it has been reported that indoleamine 2,3-dioxygenase (IDO) is essential for inhibition of parasite growth [26]. Interferon gamma induces IDO production in human cell lines but not in mouse fibroblast cells [27]. IDO production is, however, blocked by iNOS and so in human cell lines, iNOS promotes *T. gondii* growth, unlike in the mouse cells, where iNOS blocks the growth of *T. gondii* [28]. In the case of pig cells, although not fully understood, the effect of IDO on *T. gondii* growth is similar to that in human cells rather than in mouse cells [29]. Thus, TgCatJpOk4 strain might also have a high virulence in humans. Further epidemiological and molecular studies are necessary to understand the mechanism of virulence of *T. gondii* strains in humans and domestic animals.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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