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Serological Cross-Reactivity between *Sarcocystis* and *Toxoplasma* in Pigs

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INTRODUCTION

Sarcocystis, like *Toxoplasma*, is common in a wide range of domestic and wild animals(Mun, 1965; Seneviratna *et al.*, 1975; Dubey, 1976; Prestwood *et al.*, 1980; Choi *et al.*, 1987). They are both coccidian parasites belonging to the class Sporozoea of the phylum Apicomplexa. *Sarcocystis* has a mandatory two-host life cycle. The sexual stages are in the intestine of a carnivore(final host), and asexual stages are in the vascular system and musculature of a herbivore (intermediate host).

Toxoplasma has only one common primary host-the domestic cat where both sexual and asexual generations occur. In the rest of the mammalian host, *Toxoplasma* undergoes asexual reproduction.

It has recently been recognized that certain *Sarcocystis* species and *Toxoplasma* can cause severe and even fatal diseases during their acute phase of infection in intermediate host (Fayer *et al.*, 1976; Dubey, 1976; Zielasko *et al.*, 1981; Dubey *et al.*, 1981; Barrows *et al.*, 1982).

A variety of serodiagnostic tests have been used to detect serum antibodies to sarcocystosis and toxoplasmosis in men and animals, *e.g.* indirect hemagglutination (IHA) test, indirect latex agglutination (ILA) test, enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) test (Jacobs and Lunde, 1957; Kelen *et al.*, 1962; Fletcher, 1965; Suh and Jang, 1972; Suzuki *et al.*, 1980; Choi *et al.*, 1982).

Fayer and Lunde (1977) detected circulating antibodies in cattle infected with *Sarcocystis* by IHA test. In case of experimental infection with *Sarcocystis* spp. to mice, pigs and sheep, ELISA employing conjugates specific for the relevant host immunoglobulin classes M and G were then evaluated for use in serodiagnosis of these infections(O'Donoghue and Weyreter, 1984). Weyreter and O'Donoghue (1982) also examined class-specific antibody responses in pigs following immunization and challenge with sporocysts of *Sarcocystis miescheriana* (*S. suicanis*) by using IFA test and ELISA. Elamin (1985) investigated on the changes of serum proteins and immunoglobulins in pigs experimentally infected with *S. suicanis* by means of IFA test.

Sarcocystis suicanis and *Toxoplasma gondii* belong to the same Family Sarcocystidae and pigs may be infected with both parasites. These parasites produce cysts in tissue of pigs, while it is difficult to differentiate each clinical sign of sarcocystosis and toxoplasmosis in this animal (Farrell *et al.*, 1952).

This study was undertaken to measure IgG class-specific anti-*Sarcocystis* and anti-*Toxoplasma* antibodies in pigs experimentally infected with *Sarcocystis* sporocysts and *Toxoplasma* oocysts respectively, and to examine cross-reaction between *Sarcocystis* and *Toxoplasma* by using IFA test and ELISA.

MATERIALS AND METHODS

1. Animals

Nine Yorkshire cross weanling pigs (5~7kg)

were obtained from confinement rearing facility previously found to be free of *S. suicanis* and *T. gondii*. Pigs were divided without bias into three groups, each group consisting of three pigs. The groups of pigs were housed in separate concrete rooms with self feeders and water in the swine isolation facility, Veterinary Research Farm of The University of Georgia. The first group is for *Sarcocystis* infection, the second group for *Toxoplasma* infection and the third group for control.

2. Parasites

Sporocysts of *S. suicanis* for infection of pigs were obtained by scraping intestinal mucosa of dogs experimentally infected with *S. suicanis*. The scrapings were digested in approximately 10 volumes of a digestion solution (10 g of pepsin, 1,660 ml of tap water, and 13.3 ml of concentrated HCl), incubated at 37°C for 15 minutes, centrifuged, and decanted. The collected sporocysts were suspended in Hank's balanced salt solution and washed several times. Each of 3 pigs in one group was given 1.5×10^6 sporocysts of *S. suicanis* orally (*via* gastric intubation).

T. gondii oocysts were collected from the feces of experimentally infected cats by means of Dubey *et al.* (1972). Each of 3 pigs in another group was given orally 10,000 sporulated oocysts of the TS-2 strain of *T. gondii*.

All pigs were bled from the anterior vena cava prior to initiation of the infection and at week intervals thereafter until the experiment was terminated 62 days post infection. Blood samples were allowed to clot at room temperature, and the sera were decanted and centrifuged at 3,000 rpm for 10 minutes. The sera were frozen at −70°C until later analysis.

3. Antigen for IFA test

Bradyzoites of *S. suicanis* were recovered from experimentally infected pork 62 days post infection. Infected pork was ground and digested in the digestion solution for 2 hours at 37°C. After filtering through cheesecloth, the suspension was centrifuged and the sediment was saved. After washing in saline, the bradyzoites were cleaned by density gradient centrifugation using Percoll (colloidal PVP coated silica, Sigma Chem. Co.).

Toxoplasma tachyzoites of the RH-strain were obtained by serial passage in the peritoneal cavity of mice. After washing 3 times with PBS, bradyzoites of *S. suicanis* and tachyzoites of *T. gondii* were used as particulate antigen by layering on a multispot slide (Cell-Line Associates, Newfield, NJ). The slides were air dried and maintained at −60°C until used.

4. Antigen for ELISA

Antigen for ELISA was prepared by Gasbarre *et al.* (1984). The bradyzoites and tachyzoites were ruptured by repeated freeze-thawing three times followed by sonication. The suspension was centrifuged at 20,000g for 30 minutes. The supernatant was collected and dialysed overnight at 4°C against PBS, pH 7.2 using a dialysis membrane with a 6,000 to 10,000 molecular weight cut off. The dialyzed antigen was stored at −60°C. The protein concentration was estimated with the Bio-Rad coomassie blue dye-binding assay.

5. IFA test

Ten microliters of each serum diluted in two fold were applied to well containing an antigen on the slide. The slides were then incubated in a humidified chamber for 30 minutes at room temperature, washed in PBS (pH 7.6) for 30 minutes with mild stirring, and layered with 10 μl Protein A-fluorescein isothiocyanate (FITC) labelled conjugate (Sigma Chem. Co.) in Evans' blue (1 : 400 conjugate, 1 : 30,000 Evans' blue) for 30 minutes by means of Elamin (1985). The slides were placed in PBS, stirred gently and washed for 1 hour. Each slide was air dried, mounted with a cover glass using buffered glycerol (pH 9.0), and examined with an epifluorescent microscope (American Optical Co.).

The reaction was considered negative if the organisms showed no fluorescence, fluoresced reddish-purple, or when only the anterior end of the organisms fluoresced bright yellow-green with no extension of a yellow-green fluorescence around the posterior end. The reaction was considered positive when yellow-green fluorescence extended around entire periphery of the

organism. The titer was determined as the end point dilution, after which the serum showed negative reaction on subsequent dilution.

6. ELISA

Enzyme immunoassay using class-specific conjugates was used to quantitate the IgG antibodies present in the test serum pools, as follows: 1) Wells of polyvinyl microtiter plates (Dynatech Immulon) were coated with 100 μl of soluble antigens at a protein concentration of 10 μg/ml in 0.05M carbonate coating buffer (pH 9.6) overnight at 4°C. The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). 2) Each well was post-coated with 100 μl of PBS containing 1.5% bovine serum albumin (BSA) for 1 hour at 36°C and the plates were washed 3 times with PBS-T. 3) The test serum pools were titrated in PBS-T (giving double-dilutions from 1/2 to 1/1024) and a 100 μl aliquot of each dilution was added to the wells. The plates were incubated for 1 hour at 36°C and then washed 3 times with PBS-T. 4) Rabbit anti-swine IgG conjugated with horseradish peroxidase (Kirkegaard & Perry Co.) was diluted to its working dilution 1 : 9,000 with PBS containing 4% BSA and 100 μl aliquots were then added to the relevant test wells. The plates were incubated for 1 hour at 36°C and then washed 3 times with PBS-T. 5) Each well then received 100 μl of an enzyme substrate consisting of a peroxidase substrate system. The plates were allowed at room temperature for 20 minutes.

Absorbance values were then read spectrophotometrically at 405 nm. The resulting color change in each well was determined with a plate reader (Bio-Rad).

The optical dilutions of the antigen concentration and all of the reagents used were determined in preliminary block titrations. The optimal concentrations of reagents were determined on the basis of the best resolution obtained between positive and negative control sera. The end point of the IgG-ELISA was the highest serum dilution that exhibited twice the reactivity of mean absorbance of ten wells containing the negative control serum. Under the experimental conditions described above, the negative control serum gave absorbance value of $\leq$0.477 to both antigens and typical positive absorbance value of homologous reaction was 1.542 in sarcocystosis and 1.584 in toxoplasmosis.

RESULTS

IFA test:

The serological responses examined by the class-specific IgG-IFA test in pigs infected with *Sarcocystis* and *Toxoplasma*, respectively, are shown in Fig. 1. The reciprocal titers are mean values of 3 pigs. Antibodies to *Sarcocystis* were detected first at 2 weeks post infection. The titer increased in a linear fashion throughout the course of the experiment and reached its maximum (1 : 1024) terminally.

Antibody production to *Toxoplasma* was detected first at 2 weeks post infection. The titer reached its maximum at 6 weeks post infection and decreased thereafter, until the pigs were sacrificed at 9 weeks post infection. Cross-reaction titer of pig serum infected with *Toxoplasma* against *Sarcocystis* antigen appeared up to 1:16. The serum titers of control group were below 1 : 4.

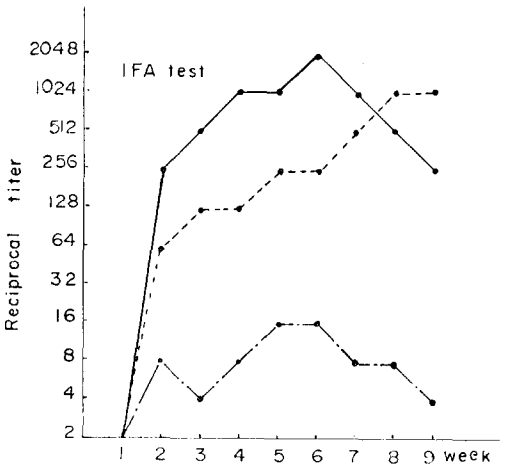

Fig. 1. Dynamics of IgG antibody of pigs infected with *Sarcocystis*(···)and *Toxoplasma*(—) to homologous antigens, and *Toxoplasma*(-·-) to *Sarcocystis* antigen by IFA test.

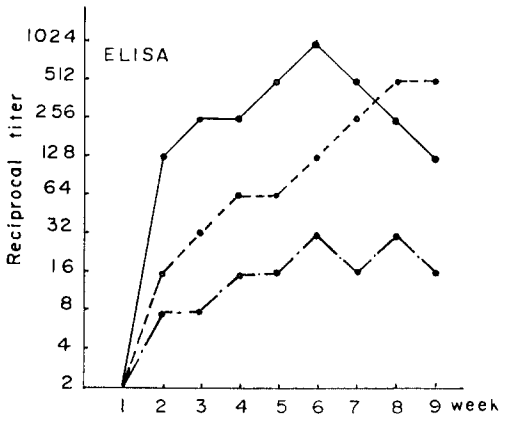

Fig. 2. Dynamics of IgG antibody of pigs infected with *Sarcocystis*(···) and *Toxoplasma*(—) to homologous antigens, and *Toxoplasma*(-·-) to *Sarcocystis* antigen by ELISA.

ELISA:

Sera from pigs infected with *Sarcocystis* and *Toxoplasma* respectively, were tested in the class-specific immunoassay. The kinetics of IgG antibody responses are given in Fig. 2. IgG antibodies of pigs to *Sarcocystis* appeared 2 weeks after infection and the titers slowly increased until the pigs were sacrificed. IgG antibodies of pigs to *Toxoplasma* appeared at comparatively higher level(1 : 128) 2 weeks post infection and reached its maximum titer(1 : 1024) 6 weeks post infection and decreased thereafter. Cross-reaction titer of pigs infected with *Toxoplasma* to *Sarcocystis* antigen appeared up to 1:32. Control serum titers were below 1 : 8.

DISCUSSION

Sarcocystis spp. are a cause of severe and even fatal disease in several domestic animals, such as pigs, cattle, sheep and goats (Barrows *et al.*, 1982; Dubey, 1976; Fayer *et al.*, 1976). The prevalence of anti-*Sarcocystis* antibodies in pigs has been reported to be 3.4%~32% in the United States (Prestwood *et al.*, 1980; Seneviratna *et al.*, 1975). *Sarcocystis* cysts are often found in tissue sections of pigs and cattle but the prevalence is unknown in Korea.

A serological test is needed for differential diagnosis between sarcocystosis and toxoplasmosis in pigs, because the clinical signs of both diseases are similar in both the subclinical and acute manifestations. There are many reports of immunological study on pigs experimentally infected with *Sarcocystis* and *Toxoplasma*, but few cases in the cross-reaction test between *S. suicanis* and *T. gondii* (Andrade and Weiland, 1971; Elamin, 1985; O'Donoghue and Weyreter, 1983 & 1984; Weyreter *et al.*, 1984; Zielasko *et al.*, 1981).

In this paper, the class specific anti-*Sarcocystis* IgG antibodies were first detected 2 weeks post infection in the pigs infected with *S. suicanis* by means of IFA test and ELISA, and the titer increased in a linear fashion until the experiments were finished 9 weeks post infection. The first detected time and kinetics of IgG antibody are similar to the results of Weyreter and O'Donoghue(1982), and Fayer and Lunde(1977), but some different values have appeared. It is guessed that these differences in the results are derived from the number of infected sporccysts, the time after infection, and physiological condition of the host.

Anti-*Sarcocystis* IgG antibody was detected 25~35 days post infection by means of IFA test and ELISA in pigs experimentally infected with 1,000 sporocysts of *S. miescheriana* and the titer reached maximum 50~84 days post infection and remained at plateau levels until the end of the observation period(Weyreter and O'Donoghue, 1982). The development of anti-*Sarcocystis* IgG antibody in pigs infected with 50 sporocysts of *S. miescheriana* three times a week was positive between day 14 to 42 and increased after challenging infections(Weyreter and O'Donoghue, 1982; Weyreter *et al.*, 1984). In pigs inoculated each with 1,800 sporocysts of *S. suicanis*, IFA-titers became positive after 25 to 28 days and reached maximum values of 1 : 40 to 1 : 80 between day 92 and 106 post infection(Zielasko *et al.*, 1981).

In this paper, antibody production against *Toxoplasma* in pigs experimentally infected with *T. gondii* oocysts was first detected at 2 weeks

post infection. The titer reached maximum 6 weeks post infection and dropped thereafter. Those results are the same as the experiment of Fletcher(1965).

The IFA test was performed by the standard procedure with the exception that a FITC-labeled protein A conjugate was used. The ELISA was used as described by O'Donoghue and Weyreter(1984) with some exceptions. In order to reduce non-specific reaction, each well was post-coated with PBS containing 1.5% BSA and rabbit anti-swine IgG conjugated with horseradish peroxidase was diluted to its working dilution of 1 : 9,000 with PBS containing 4% BSA. In this experiment, sulfuric acid or sodium hydroxide was not used after adding substrate and the reactions were read by measuring their absorbance values on a defined time.

The soluble *S. suicanis* antigen was cross-reacted with anti-*Toxoplasma* antibody raised in pigs at titer of up to 1 : 16 on IFA test and up to 1 : 32 on ELISA. Antigen from *Sarcocystis* bradyzoites from cattle did not cross-react with the serum of man naturally infected with *T. gondii*(Lunde and Fayer, 1977). Sera from 2 calves inoculated 28 to 45 days previously with 5×10^4 *T. gondii* oocysts showed no reaction with *S. bovicanis* antigen by means of ELISA (Gasbarre *et al.*, 1984).

By means of the micro-agar-precipitation, indirect fluorescent antibody test, and Sabin-Feldman test, investigations were carried out on the serological relationship between *Eimeria nieschulzi*, *E. scabra*, *E. polita*, *Isospora felis*, *Sarcocystis tenella* and *T. gondii*.

Using the micro-agar-precipitation, cross-reactions could be demonstrated between the different *Eimeria*-species, *Eimeria* and *Toxoplasma*, as well as *Isospora felis* and *Toxoplasma* oocysts. This last reaction was, however, very weak. Using *Sarcocystis* and *Toxoplasma* antigens in IFA test and Sabin-Feldman test only specific reactions occurred(Andrade and Weiland, 1971).

It is interesting to compare the kinetics of anti-*Sarcocystis* IgG antibody with anti-*Toxoplasma* IgG antibody in pigs. Both anti-*Sarcocystis* and anti-*Toxoplasma* IgG antibodies were detected 2 weeks post infection. Anti-*Sarcocystis* antibody increased until this experiment was finished 9 weeks post infection, while anti-*Toxoplasma* antibody began to decrease from 6 weeks post infection. It is suggested that differences in the kinetics of IgG antibody to *Sarcocystis* and *Toxoplasma* are caused from different construction of cyst wall formed in tissue. Both cysts begin to mature from 5~8 weeks post infection. When the cyst of *Toxoplasma* is completely matured, the antigen in the cyst can not affect the host whereas the cyst of *Sarcocystis* can affect the host even after it has been matured. This hypothesis is agreed to that high titer of IgG antibody to *Sarcocystis* in pigs was detected after tissue cysts had been formed.

SUMMARY

The development of antibody titers and cross-reaction between *Sarcocystis* and *Toxoplasma* were investigated by means of IFA test and ELISA in pigs experimentally infected with 1.5×10^6 *S. suicanis* sporocysts and 10,000 *T. gondii* oocysts, respectively.

The intact and soluble *Sarcocystis* antigens were prepared from the bradyzoites harvested by peptic digestion of infected pork. The intact and soluble *Toxoplasma* antigens were prepared from the tachyzoites in mouse peritoneal cavity. IgG antibodies in pigs infected with *Sarcocystis* and *Toxoplasma*, respectively were detected first at 2 weeks post infection on both IFA test and ELISA. The antibody titer to *Toxoplasma* reached its maximum at 6 weeks post infection and decreased thereafter. The antibody titer to *Sarcocystis* reached its maximum terminally. The cross-reaction titer in pigs infected with *Toxoplasma* against *Sarcocystis* antigen was up to 1 : 16 in IFA test and up to 1 : 32 in ELISA. The titer in control group was below 1 : 4 in both reactions.

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=국문초록=

돼지에 있어서 *Sarcocystis*와 *Toxoplasma* 感染의 血清學的 交叉反應 試驗

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돼지에 1.5×10^6개의 *Sarcocystis suicanis* sporocyst와 10,000개의 *Toxoplasma gondii* oocyst(미국 Georgia 대학교 수의과대학 保存株)를 각각 經口感染 시킨후 9주간에 걸쳐 血中 IgG 抗體의 消長과 *Sarcocystis*와 *Toxoplasma* 사이의 血清學的 交叉反應性을 IFA 검사와 ELISA法을 이용하여 검사하였다. IFA검사와 ELISA를 위한 *Sarcocystis*의 全蟲體 抗原과 水溶性 抗原은 感染豚의 筋肉을 인공소화액으로 소화시켜 준비하였다. *Toxoplasma*의 全蟲體 抗原과 水溶性 抗原은 감염 mouse 복강에서 蟲體를 分離하여 사용하였다.

*Sarcocystis*와 *Toxoplasma*를 각각 인공감염시킨 豚血清의 IgG 抗體는 IFA검사와 ELISA에서 모두 감염 2주째에 처음으로 檢出되었으며 *Toxoplasma*에 대한 抗體價는 감염 6주째에 最高値에 도달하였다가 그 이후에는 下降하였다. *Sarcocystis*에 대한 抗體價는 감염 9주까지 서서히 上昇하였다. *Toxoplasma*에 감염된 豚血清은 *Sarcocystis* 抗原에 대하여 IFA검사에서는 1：16까지 교차반응이 나타났으며 ELISA에서는 1：32까지 나타났다. 대조군의 豚血清은 모두 1：4 이하이었다.