Natural infections of pigs with akabane virus

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Abstract

Akabane (AKA) virus is considered a pathogen of herbivores in nature. However, we found that pig populations in fields were infected in Tainwan. Anisolate (NT-14) of AKA virus was obtained from pigs. The NT-14 virus was able to infect pigs by the oronasal route. Subsequently, low levels of infectious virus particles were excreted into the oronasal discharge during the stage of viremia but they were not sufficient to infect new porcine hosts via contact transmission. The prevalence of serum neutralizing antibodies to AKA virus in pig populations was investigated, indicating that approximately 75% of pigs in Taiwan were seropositve. Sows and newborn piglets have the highest titers of neutralizing antibodies. Contrarily, fattening pigs aged at approximately 20 weeks old contained the lowest titers of specific antibodies. Our results suggest that pigs in natural situations are part of the AKA virus transmission cycle.

Keywords: akabane virus; Arbovirus; Culicoides spp.; Bunyavirus; Simbu serogroup

1. Introduction

Akabane (AKA) virus is a member of the Simbu serogroup of the genus Orthobunyavirus. The virus genome consists of three unique segments of single-stranded negative-sense RNA, large (L), medium (M) and small (S), which differ in size with approximately 7, 4, and 0.86 kb, respectively (Pattnaik and Abraham, 1983; Fenner et al., 1993; Akashi et al., 1997).

AKA virus has demonstrated its replication ability in many kinds of natural host species and in several experimental animals. Based on serological evidence, herbivores includ-

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ing cattle, horses, donkeys, sheep, goats, camels and buffaloes appear to be infected in natural situations (Cybinski et al., 1978; Al-Busaidy et al., 1988). Disease cause by AKA virus in

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cattle, sheep and goats is associated with stillbirths, abortions, congenital arthrogryposis-hydranencephaly syndrome, and hydranencephaly micrencephaly syndrome (Inaba et al., 1975; harley et al., 1997; Della-Porta et al., 1977; Parsonson et al., 1981; Haughey et al., 1988; Whittington et al., 1988). Outbreaks of the disease resulting in con- genital malformations in ruminants have occurred in Japan, Australia, Israel, Turkey, Korea and Taiwan (Inaba, 1979; Shimshony, 1980; Yonguc et al., 1982; Konno et al., 1982; Liao et al., 1996a; Lee et al., 2002). Experimental animals such as chicken embryos, mice and hamsters are also susceptible to artificial infections and their infection may result in deaths or congenital deformities (Andersen and Campbell, 1978; Nakajima et al., 1979, 1980; McPhee et al., 1984; Konno et al., 1988).

AKA virus is arthropod-borne, replicating in and being transmitted by either mosquitoes or midges (*Culicoides*). Vector species concerned in virus replication and transmission have been intensely studied. In Austrlia, two species of midge, the *Culicoides nubeculosus* and *C. variipennis* (Jennings and Mellor, 1989), have been shown to support virus replication. Virus transmission is also demonstrated to be mediated via the bites of *C. brevitarsis* and *C. nebeculosus* (Doherty, 1972; Murray, 1987; Jennings and Mellor, 1989). In Japan, AKA virus was isolated from *C. oxystoma* (Kurogi et al., 1987) and from mosqutoes, including *Aedes vexans* and *Culex tritaeniorhynchus* (Oya et al., 1961).

In 2000, an isolate (NT-14) of AKA virus was obtained from a diseased pig aged at 14 weeks old in Taiwan, which afforded the opportunity to investigate the pathogenicity and seroprevalence of AKA virus in fections in pigs. Our studies revealed that most pigs in Taiwan were seropositive to AKA virus. Especially, sows and finishing pigs were under high risk of infection.

2. Materials and methods

2.1. Isolation of virus and serum neutralization test

Vero cells, a continuous cell line derived from the African green monkey, were used to isolate, replicate AKA virus and to perform the serum neutralization (SN_{50}) test. The SN_{50} test was carried out by the microtiter method (Al-Busaidy et al., 1988; OIE, 1996a). Briefly, serial two-fold dilutions of the serum from 1/2 to 1/256 were performed with maintenance medium in 50 μl volumes in plates and mixed with equal volumes (50 μl) of maintenance medium, containing 100 $TCID_{50}$ of AKA viruses, employing two wells for each serum dilution (OIE, 1996a). After incubation at 37℃ for 1 h, a volume of 100 μl of cell suspension containing a concentration of 10^6 cells/ml was added to each well. Antibody titers were determined after a 6-day incubation.

2.2. Virus identification with cross-neutralization test

To test whether the NT-14 virus was AKA virus, serological cross-neutralization test with the NT-14 and TS-C2 viruses was performed. The TS-C2 virus is a vaccine strain (Kitani et al., 2000), which has been maintained in this institute since 1991. Specific antiserum to the TS-C2 virus was raised by rabbit inoculated with a dose of $10^{6.5}$ $TCID_{50}$ of virus, reaching 1:1024 of the neutralizing antibodies. In addition, antiserum to the NT-14 virus was produced

from pig inoculated with the NT-14 virus, reaching 1:64 of neutralizing antibodies. The cross-reactivity by SN test with the TS-C2 and the NT-14 viruses and their paired serums was performed (OIE, 1996b). Viruses were diluted in maintenance medium over the range 10^{-1} to 10^{-8} in 10-fold steps. Equal volumes of antiserums diluted 1/10 were added to the diluted viruses. Mixtures were incubated at 4℃ overnight and thereafter inoculated into wells of microtiter plates with monolayer of Vero cells. Assessment was carried out 6 days later, based on the appearance of cytopathic effect (CPE).

2.3. RT-PCR and sequence analysis

Based on the S-RNA sequences of the PT-17 virus, a Taiwanese isolate from cattle (Chang et al., 1998), four specific oligonucleotide primers, F1 (forward sequence 5'-TACGCA-TTGCAATGGCAAATC-3', corresponding to residues 1-21), F2 (forward sequence 5'-AAGGGTTGCACTTGGAGTGA-3', corresponding to residues 339-418), R1 (reverse sequence 5'-AGGAAAGCTCTAGCTGCAGG-3', corresponding to residues 669-668) and R2 (reverse sequence 5'-TATAAACAATAAAATCCAAGCAGC-3', corresponding to resi- dues 786-814), were used in RT-PCR reactions and DNA sequencing. The RT-PCR re- actions were performed in a single reaction tube by a previously established protocol (Huang et al., 2001). Viral RNA was extracted from infected culture fluid, using QI- Aamp Viral RNA Mini kit (QIAGEN) by the method recommended by the manufac- turer. The amplified DNA fragments were sequenced by the direct sequencing method, using BigDye™ Terminator Cycle Sequencing kit and ABI 3500 DNA sequencer (Applied biosystems).

Phylogenetic analysis on the S-RNA sequences was performed by using LaserGene Bio-computing Software Package (DNASTAR, 1997). Briefly, nucleotide sequences obtained in this study corresponding to sequences 22-787 bp of the PT-17 S-RNA and those available from the international DNA data bank (NCBI) were maximally aligned using MegAlign pro- gram in the DNASTAR package. The phylogenetic tree was constructed using CLUSTAL algorithm.

2.4. Animal inoculation and in-contact transmission

A total of 12 seronegative ($\leqq$1:3 in SN_{50} titer)pigs aged at 4 weeks old were used to study the susceptibility, pathogenicity, virus replication, virus transmission, development of antibodies and virus excretion following AKA virus inoculation. Five of the 12 pigs were inoculated with a dose of $10^{6.5}$ $TCID_{50}$ in 5 ml of the NT-14 virus via the oronasal route. Five other pigs were inoculated with the same dose of virus via the intra-muscular rout. In addition, two pigs, which did not receive virus, were housed together with the inoculated pigs to study the contact transmission. Pigs were sampled daily for the oronasal discharge, whole blood, serum and feces for virus isolations and for testing viral antibodies. Five of the infected pig were sacrificed at the 4th, 6th, 9th, and 14th days postinfection
for histopathological examination and virus recovery. The selected specimens including

tonsils, spleen, lung, liver, kidney, cellebrum, cellebellum, small intestine, large intestine, lymph nodes, thymus and saliva gland were subjected to virus isolation with Vero cells. Then, the recovered viruses were tested with RT-PCR to identify the AKA specific nu- cleotides and their abilities to neutralize the AKA-specific antiserum. Parts of tissue sam- ples were also fixed in 10% formalin, embedded in paraffin wax and routinely processed for histopathology.

2.5. Serum samples

To investigate the prevalence of anti-AKA antibodies in pig populations, serum samples were randomly collected from 21 pig farms and 6 abattoirs attributing over northern to southern Taiwan during August to October 2001. Serum samples contained 150 of sows and 561 of newborn to 20-week-old pigs. In addition, 377 samples were collected from the finishing pigs in 6 abattoirs. These serum samples were tested for the titers of neutralizing antibodies with the NT-14 virus. A serum was taken as positive for antibody titers $\geqq$1:4 (Della-Porta et al., 1976). The average SN titers were derived from the sum of the titers at the same age divided by the animal number.

3. Results

3.1 Virus isolation and identifications

In 2000, three diseased pigs aged at 14 weeks old with clinical signs of convulsions and diarrhea were submitted to this institute. Two distinct viruses were isolated from different pigs. One of the two viruses has been identified to be the porcine teschovirus and the other was the NT-14 virus obtained from tonsils of a pig. The NT-14 virus has ability to infect a wide range of cultured cells including swine thyroid (ST), baby hamster kidney (BHK-21), Vero, rabbit kidney (RK-13), embryo bovine testis (EBT) and embryo bovine kidney (EBK) cells, reaching titers of $10^{6.0}$ to $10^{7.0}$ $TCID_{50}$/ml.

The NT-14 virus was observed with EM and showed Bunyavirus-like particles. RT-PCR using random primers (data not shown) was performed and obtained fragment with se- quences similar to the S-RNA of AKA virus. A serum cross-neutralization test using the TS-C2 virus and its paired serum was performed to identify the NT-14 virus. Results indi- cated that the anti-TS-C2 rabbit serum (SN50 = 1024) could neutralize $10^{4.57}$ $TCID_{50}$ of the TS-C2 virus and neutralize $10^{4.67}$ $TCID_{50}$ of the NT-14 virus (Table 1). The anti-NT-14 pig serum (SN_{50} = 64) could neutralize $10^{2.96}$ $TCID_{50}$ of the NT-14 virus and neutralize $10^{3.0}$ $TCID_{50}$ of the TS-C2 virus (Table 1).

Table 1
The cross-neutralization test of two virus isolates and their paired serums

Virus strain	Rabbit anti-TS-C2 serum(SN = 1024)	Pig anti-NT-14 serum (SN = 64)
NT-14	$10^{4.67}$ $TCID_{50}$	$10^{2.96}$ $TCID_{50}$
TS-C2	$10^{4.57}$ $TCID_{50}$	$10^{3.0}$ $TCID_{50}$

SN: serum neutralization test.

3.2 RT-PCR amplifying the S fragment and the study of phylogenetic tree

By using specific primers to the AKA PT-17 virus, most of the nucleotide sequences (776 bp)

of the S-RNA were obtained from the NT-14 virus. The nucleotide sequence was submitted to the GenBank in NCBI with the accession number AF529883. Sequence iden- tity of the S-RNA between the NT-14 and the PT-17 Taiwanese isolates was approximately 99.6%. Phylogenetic analysis using S-RNA nucleotide sequences from the GenBank in- cluding 16 Japanese isolates, 2 Australian isolates and the 2 Taiwanese isolates, indicated that the 20 AKA viruses could be divided into three major clusters. The first cluster (group I) (Fig. 1) included isolates of the Iriki, KC15X84, KC-04Y84, KC-12X84, KS-90-2, FO-90-4, MZ-90-1, and KM-29X82, which were obtained from Japan. The two Taiwanese isolates, PT-17 and NT-14, clustered into the group I were closely related to the Iriki and KC isolates with approximately 98.7% similarities. The second cluster (group II) consisted of M-171, OBE-1, NBE-9, KT3377, NS-88-1, ON-89-2, JaGAr39 and FO-90-3, was isolates from Japan (Fig. 1). The two Australian isolates (B8935 and R7949) were placed in the third cluster (group III, Fig. 1), showing considerable divergence from all of the Japanese and Taiwanese isolates.

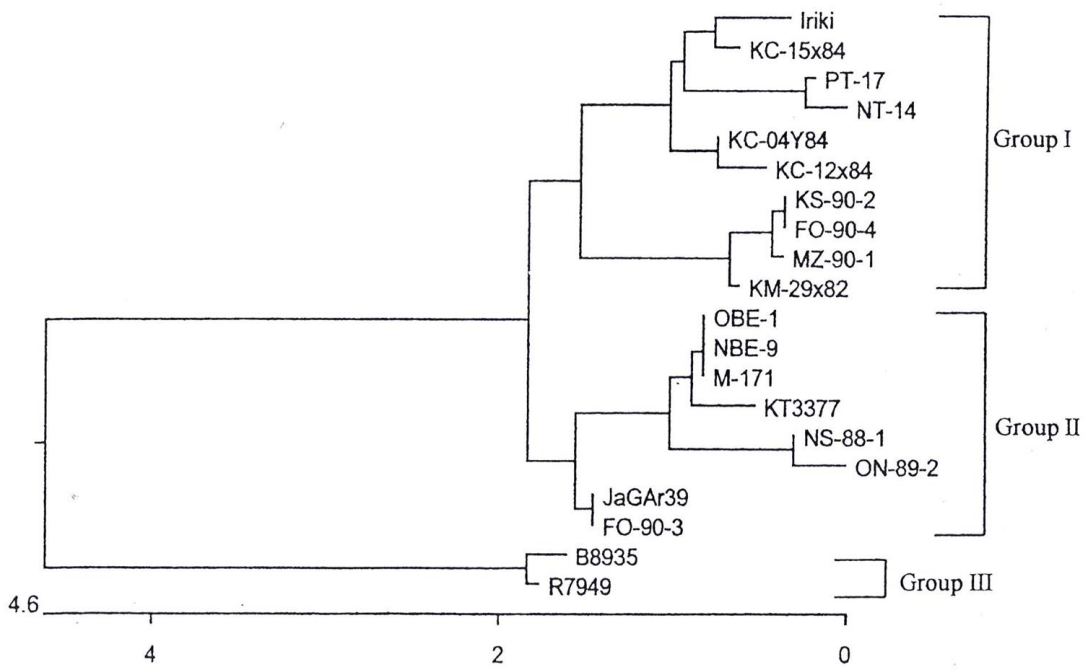

Fig. 1. Phylogenetic tree showing genetic relationship between the 20 AKA S-RNA sequences. The tree was constructed by using the MegAlign programs of the DNASTAR package. The accession number from GenBank (NCBI): Irki AB000863; KC-15C84 AB000861; PT-17 AF034940; NT-14 AF529883; KC04Y84 AB000862; KC-12X84 AB000860; KS-90-2 AB000872; FO-90-4 AB000871; MZ-90-1 AB000868; KM-29X82 AB000859; M-171 AB000858; OBE-1 AB000851; NBE-9 AB000855; KT3377 AB000857; NS-88-1 AB000864; ON-89-2 AB000867; JaGAr39 AB000852; FO-90-3 AB000870; B8935 AB000853; R7949 AB000854.

Table 2

Viral recovery from tissue samples of pigs after the NT-14 virus inoculation

Organ	Animal no. (sacrificed day postinfection)				
	1(4)	2(6)	3(6)	4(9)	5(14)
Tonsil	+	+	+	+	+
Spleen	−	+	−	−	−
Lung	+	−	+	−	−
Liver	−	−	−	−	−

Kidney	−	−	−	−	−
Cellebrum	+	−	−	−	−
Cellebellum	+	−	−	−	−
Small intestine	+	−	−	−	−
Large intestine	−	−	−	−	−
Lymph-node mixture	+	+	−	−	−
Thymus	−	+	−	−	−
Saliva gland	−	−	+	−	−
Whole blood	+	+	+	−	−

3.3. Aninmal inoculations

A total of 12 seronegative pigs were used to study the pathologic lesions, virus replica- tion, virus excretion, antibody development and contact transmission following inoculation of the NT-14 virus. Five of 10 infected pigs were necropsied at the 4th, 6th 9th and 14th days for testing virus recovery from tissues and for examining histopathologic lesions. There were no gross pathological lesions detected in the infected pigs. However, with microscopic examinations, two infected pigs sacrificed at the 4th and 6th days showed mild nonsuppra- tive encephalitis and vasculitis infiltrated with lymphocytes on brains (data not shown). For testing the virus recovery, the infectious viruses were recovered with varying frequency from the brains, small intestine, thymus, spleen, saliva gland, lymph node mixtures, lungs, tonsils and the whole blood during the 4th and 6th days postinfection (Table 2). The infec- tious viruses were continually recovered from the tonsils until the 14th days postinfection (Table 2). Infectious virus was recovered from the oronasal discharge at the 4th and the 6th days in 2 of 10 infected pigs (data not shown) but the infectious virus was not obtained from the feces during the whole period of the experiment.

The kinetics of antibody development in the infected and the contact-transmission pigs were examined. All recipients receving virus via IM or oronasal routes responded by antibody development (Fig. 2). By IM route, neutralizing antibodies were detected at the 3rd to 5th days and the antibody titers reached $\geqq$724 at the 9th day (Fig. 2). By oronasal route, neutralizing antibodies were detected at the 4th to 6th days and the titer reached $\geqq$724 at the 6th day(Fig 2). The contact-transmission pigs did not seroconvert to positive during the period of the experiment (Fig. 2).

3.4 Prevalence of neutralizing antibodies to AKA virus in pigs

To investigate the prevalence of specific antibodies against AKA virus in pigs, a national survey of sera on pig populations was carried out. The results showed that approximately

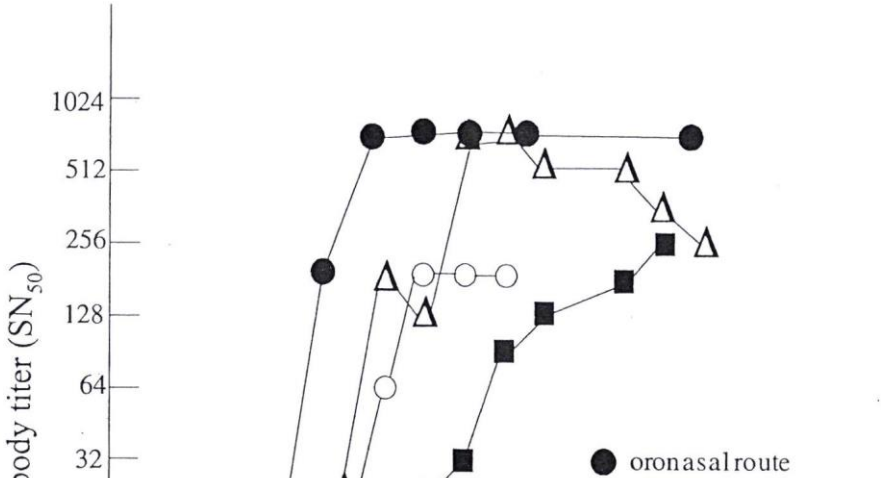

Fig. 2. Kinetics of antibody development following the NT-14 virus inoculation. Each seronegative pig was inoculated with a dose of $10^{6.5}$ $TCID_{50}$ of the NT-14 virus via the oronasal or intra-muscular routes. Serums from the infected and in-contact pigs were collected every day postinfection for titration of neutralizing antibodies.

75% (816/1088) of pigs present seropositive (≧4) to AKA virus (Fig. 3). The sows and newborn piglets had the highest levels of neutralizing antibodies, the average SN titer was 392.2 in the sows and 383.2 in the newborn piglets. The average titer was 140.2 in the 3-week-old pigs and 42.5 in the 6-week-old pigs. For the 9 and 12 weeks old pigs, the average titers were 14.9 and 30.9. For the 14 and 20 weeks old pigs, the average titers were 46.2 and 14. However, the average titer on the finishing pigs was increased to 91.6. In addition, the sow showed 99.4% seropositive. The 3-week-old piglets present 98.4% seropositive. The 20-week-old pigs only showd 17.2% seropositive and the finishing pigs were 71.4% serpositive.

4. Discussion

Previous studies (Cybinski et al., 1978; Al0Busaidy et al., 1988) have indicated that the herbivores including cattle, sheep, giraffes, horses and goats are the natural hosts in the AKA virus infection cycles. However, the virus was only obtained from infected cattle or ovine fetuses (Della0Porta et al., 1977; Akashi et al., 1997). Our study is the first repot on

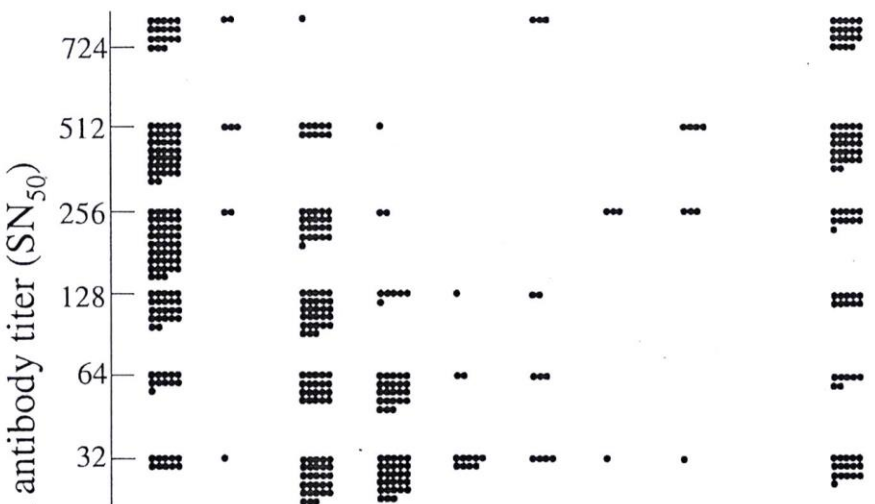

Fig. 3. Prevalence of neutralizing antibodies to AKA virus in pigs. A total of 1088 serums were collected from 21 pig farms and 6 abattoirs attributing in Taiwan. Serums from pig farms include sows (S) and 0, 3, 6, 9, 12, 14, 20 weeks old pigs. Serums of the finishing pigs (F) were collected from abattoirs. These serum samples were tested for SN_{50} titers. Antibody titers were determined from 4-724-fold.

The isolation of AKA virus from pigs. By using SN test (Table 1) and nucleotide sequencing of the S-RNA (Fig. 1), we have identified that the NT-14 virus isolated from pigs is an AKA virus. Serological investigations (Fig. 3) support that infection of AKA virus in pigs is not an accident but the virus may persist in various species of hosts and causes endemic infection in animals, which include pigs.

We have studied the NT-14 virus in pigs to characterize the pathogenicity, the virus replication, the viral excretion and infection routes. Although the histopathologic lesions were only observed in 2 of the 10 infected pigs aged at 4 weeks old with mild nonsupprative encephalitis, evidence showed that pigs were susceptible to AKA virus and supported virus replication (Table 2). One interesting finding in our studies was that high levels of AKA virus were able to infect pigs via the oronasal route and that active virus particles were recovered from the oronasal discharge in 2 of 10 the 10 infection pigs (data not shown). This result indicates that high levels of virus particles are not arthropod-dependent for infection. However, transmission via direct contact did not occur (Fig. 2). The possible explanation was that levels of active virus particles excreted into the oronasal discharge were too low to infect new hosts. We have detected that virus levels during viraemia stage were less than 10^2 $TCID_{50}$ in per ml of the whole blood (data not shown). Therefore, vectors (such as
mosquitoes or *Culicoides*) may act as a role in passing the barriers of virus concentrations and in directly injecting the active virus particles into hosts or vectors may amplify the active virus particles to infect (Jennings and Mellor, 1989; Allingham and Standfast, 1990). AKA viruses obtained from cattle (PT-17 isolate) and pigs (NT-14 isolate) in Taiwan have showed similar nucleotide sequences (99.6% similarities in the S-RNA). In addition, phylogenetic studies using the virus isolates from the GenBank and the two Taiwanese isolates (PT-17 and NT-14) have been grouped into three clusters (Fig. 1), which was consistent to previous studies by others (Akash et al., 1997; Chang et al., 1998). In the dendrogram tree analysis, the PT-17 and NT-14

viruses have shown the close relationship in evolution (Fig. 1). These results indicated that infections in pigs and in ruminants may be caused by the same virus.

Vectors of the *Culicoides* and mosquito species mediated in AKA virus infection have been intensely studied. Taiwan is located in the subtropical region in geography. Rainly season with ≧108 mm monthly rainfall and ＞25℃ temperature maintains from May to October. These weather conditions favor the proliferation of insect vectors, such as *Culi- coides* and mosquito species (Hsu et al., 1997). In Taiwan, there are more than 50 *Culicoides* species endemic (Lien and Chen, 1982), including *Culicoides brevitarsis*, *C. nipponensis*, *C. orientalis* and *C. oxystoma*, which were considered to mediate in AKA virus transmission (Kurogi et al., 1987; Doherty, 1972; Murray, 1987; Jennings and Mellor, 1989). A previous investigation has shown high levels of seroprevalence (96%) to AKA virus in Taiwan cattle (Liao et al., 1996b). In addition, mosquito vectors mediated in transmission of AKA virus including the *Aedes vexans* and *Culex tritaeniorhychus* (Oya et al., 1961) were normal flora in Taiwan (Lien, 1968). By using artificial inoculation, we have characterized the replication and susceptibility of the NT-14 virus in pigs. Summarized those physical conditions and animal studies, if pigs were susceptible to AKA virus, they were exposed under high risk of infection in Taiwan. Based on this study, we suggest that pigs should be considered as a member in the virus-host-vector circulated cycle.

Serological investigations in pig populations sampling from the whole ages and the whole region of Taiwan have revealed that approximately 75% of pigs were seropositive to AKA virus (Fig. 3). Interestingly, the proportion of seropositive pig displayed decreased levels with the increase of ages in the fatting pigs (Fig. 3). Sows and newborn piglets had the highest levels of specific antibodies and the 20-week-old pigs had the lowest levels of antibodies. These studies revealed that the high levels of antibodies in the newborn to 3-week-old pigs were most possibly maternal antibodies, which were passed from the mothers. The maternal antibodies decreased to minimum levels (17.2%) in the 20-week-old pigs, which was consistent with the decreased antibody levels in the ruminants (Al-Busaidy et al., 1988). However, serums collected from the finishing pigs in the abattoirs have shown the increased proportion of seropositive animals (71.4%) (Fig. 3). This result suggest that pigs aged after 20 weeks old may expose to high risk of virus infection

Our studies have revealed that pigs may be important in the AKA virus infection cycle. However, in experimental pigs, the NT-14 virus would not cause observed lesions in the 4-week-old pigs, which was consistent to the situations of ruminants infected by AKA virus. AKA virus only causes lesions on the first third of pregnancy in ruminants (Kurogi et al., 1977; Parsonson et al., 1975). We do not know whether the NT-14 virus possesses the ability to cause lesions in the pregnancy in pigs.

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赤羽病毒在豬隻的自然感染

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摘要：

赤羽病毒(Akabane virus)一直都被認為是只會自然感染草食獸的病原，但是我們發現遭到先在台灣的豬群也遭感染。首先我們從豬病材中分離到一株赤羽病毒(NT-14)，此病毒可以經由口鼻途徑感染豬隻。以人工接種方式感染的豬可從口鼻分泌物中排出低濃度的病毒顆粒，但排出的病毒不會感染同藉由接觸而感染同居的豬隻。我們也調查了豬隻對於Akabane病毒的血清中和抗體盛行率，發覺台灣豬群約75%為抗體陽性，其中以母豬的中和抗體及初生小豬的最高而

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2 0 週 齡 肥 育 豬 抗
體 力 價 最 低 。 我 們
的 結 果 建 議 豬 隻 是
赤 羽 病 毒 自 然 感 染
及 病 毒 傳 播 環 中 的
宿 主 之 一 。