The isolation of Akabane virus (Iriki strain) from calves in taiwan

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ABSTRACT Nonsuppurative encephalitis in calves aged 4-12 months, cow abortion and fetal deformities were endemic in dairy farms in Taiwan in recent years. A virological investigation emphasizing on Arthpodborn virus (Arbovirus) was conducted. Total of 11 strains of Akabane virus were isolated from endemic districts between June and July of 1992. Among them, seven viruses were isolated from blood samples of 15 calves showing nervous signs. Another 4 Akabane viruses were isolated from clinically healthy calves from three of six dairy farms investigated. All the six investigated farms had a recent history of abortion and fetal deformities. The isolates caused prominent cytopathic effects in HmLu－1 cells and could reach a high virus titers (5×10^6 $TCID_{50}$/ml). As demostrated by a cross neutralization test, the isolates had identical antigenicity to Iriki strain of Akabane virus, but were antigenically more distant to JaGar－39 and OBE－1 strain of Akabane virus. This is the first report on the isolation of Akabane virus in Taiwan, and also the second report on the isolation of Iriki virus in the world.

Key words: Akabane virus, epizootiology, Iriki virus.

INTRODUCTION

Akabane virus has been known to cause outbreaks of abnormal deliveries in cattle, such as abortions, stillbirths and calf deformities noted as congenital arthrogryposis-hydranencephaly (AH) syndrome (KUROGI *et al.* 1975, 1976, 1977). Akabane virus was originally isolated from mosquitoes, Ades vexans and Culex tritaeniorhychus, in Japan in summer of 1959 (MATSUYAMA *et al.* 1960, OYA *et al.* 1961). "Akabane" is the name of the village where the virus was first isolated. Subsequent serological studies have classified Akabane virus in the Simbu group (TAKAHASHI *et al.* 1978), one of the serological group in the family Bunyaviridae (PORTERFIELD *et al.* 1976).

During the outbreak in Japan in 1972～74, the virus was isolated from naturally infected bovine fetuses and from blood of sentinel cows (KUROGI *et al.* 1976). The virus was also recovered from the blood

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of sentinel cattle (St. GEORGE *et al.* 1977) and sheep (DELLA – PORTA *et al.* 1977) in Australia. In Africa, Akabane virus was isolated from mosquitoes and Culicoides (INABA *et al.* 1990). Furthermore, the virus was isolated from Culicoides oxystoma in Japan (KUROGI *et al.* 1987) and from C. brevitarsis in Australia (St. GEORGE et al. 1978). Seasonal occurrences of congenital AH syndrome in cattle, sheep and goat were also reported in Japan and Australia (INABA *et al.* 1990, St. GEORGE *et al.* 1978). A bigh incidence of neutralizing antibody to Akabane virus was noted in cattle of Japan (KUROGI *et al.* 1975). Other species found to have antibody were horse, goat and sheep (HAUGHY *et al.* 1988, INABA *et al.* 1990). There were also reports on successful virus isolations and positive serological results from cattle and other domestic animals in many southeast Asian countries, the Arabian Penisula, the Middle East, and Africa (AL – BUSAIDY *et al.* 1988).

Taiwan is a subtropical island where the latitude of Cancer Passes through the southern half, and many kinds of biting midges and mosquitoes have been discovered (LIEN *et al.* 1982). The biting midges, Culicoides oxystoma and C. nipponesis, in dairy farms of Pintung district are normally collected by light traps for study. C. oxystoma has been proved to be a vector of Akabane virus in Japan (KUROGI *et al.* 1987). Because of the geographical location, we have suspected that there are possible seasonal occurrences of the disease in Taiwan where the disease could be transmitted in the presence of these vectors in summer. The antibody to Bunyavirus group was first detected in cattle in Taiwan in 1989 by Lu *et al.* (1990) and the positive rate for Akabane virus was as high as 89 %. However, no virus isolation has been attempted.

In July of 1992, a disease characterized by nervous signs and lameness was observed in calves aged 4～8 months in Pintung and Taipei districts, Taiwan. Seven strains of the Akabane virus were isolated from affected calves in these two districts. In addition, there were four strains isolated from clinically healthy calves aged 4～12 months in Tainan district where the abortions and deformities had been observed in the previous year. The clinical, histopathological and virological studies of the disease are described in this report.

MATERIALS AND METHODS

Clinical and epizootic features of the natural infection :

Several calves with characteristic nervous signs and lameness were observed in two farms located in Pintung and Taipei districts respectively from late June to mid July of 1992. Some of these calves died within a few days after the onset of severe nervous signs and lameness. The early clinical symptomes observed in these calves included lassitude and anorexia, and then were nervous signs such as stretching of the legs. The calves were difficult to stand, and fever was detected in some sick calves.

There were several abortion and deformity cases of cows reported from six farms in Tainan district from November to December of 1991. Twenty abortion cows and six cows which had delievered deformities were found in the epizootic farms. The gestation period of these abnormal pregnancies ranged from five to seven months in cows. Some blind of lame calves were also found in these farms.

Histopathological examination :

In epizootic farms, four affected calves (one from Taipei and three from Pintung district) showing nervous signs were necropsied and the tissues including the brain and spinal cord, and skeletal muscles were collected for etiological and histopathological examinations.

Etiological examination :

Virus isolation was performed from necropsied calves. A 10 % homogenate of each tissue was made with Eagle's MEM (GIBCO CO.) containing antibiotics. After centrifugation at 4 ℃ for 20 minutes, the supernants of individual homogenate were collected and stored at – 70 ℃ for virus isolation. Virus isolation was also performed from the blood of suspected calves or cows of which the heparinized blood were washed in phosphate-buffer saline (PBS).

The heparinized blood samples were respectively centrifugated at 2,000 rpm for 10 minutes when they were delievered into laboratory. The plasma layer was depleted and the blood cell layer was resuspended to an original volume in PBS. The washing procedure was repeated for three times. After wash, the blood cell layer was resuspended to an original volume in PBS and was stored at − 70 ℃ for virus isolation.

After freezing and thawing, the suspended blood liquid and the supernant of homogenate were inoculated into cell culture for virus isolation. The HmLu − 1 (hamster lung continuous cell line), BHK − 21 (baby hamster kidney continuous cell line) and MDBK (bovine kindey cell line) cells were used for virus isolation. The monolayer cell culture, freshly prepared in 25 cm^2 flask, was respectively inoculated with sample for virus isolation. After culture in 5 % CO_2 incubator at 37 ℃ for 7 days, each culture fluid was inoculated into another freshly prepared cell culture. This culture process was propagated at least for three times. Isolation of a virus was determined by the appearence of a cytopathic effect (CPE) in culture or more subcultures.

The virus isolation was further confirmed by the electronmicroscopy (Hitach model H600, Japan) in CPE appeared cell cultures. The sample was prepared for electronmicroscopic investigation as described elsewhere (ITO *et al.* 1979). The tissues used for virus isolation were also examined for possible bacterial pathogens. Each saample was inoculated on a blood agar and tryptic soy agar plates under aerobic and anaerobic condition at 37 ℃.

Viruses and antisera :

The strain of Iriki (given by National Institute of Animal Health, Japan) and PT − 17 (Taiwan isolate) of Akabane virus were respectively subcultured in HmLu-1 cell, and viral titer measured by the simultaneous inoculation method was 5×10^6 $TCID_{50}$/ml.

Antisera of Iriki, JaGar − 39 and OBE − 1 were also given by National Institute of Animal Health, Japan. The antiserum of PT − 17 was prepared from rabbits which had been byperimmunized with PT − 17 isolate.

Serological examination :

Sera were collected on the day of sacrifice from necropsied calves with nervous signs and from cohabitating calves in the epizootic farms in Pintung and Taipei districts. In the epizootic farms located in Tainan district, sera were collected monthly from November of 1991 till July of 1992 from cows which had previous either normal delivery or abortion.

For neutralization test, all serum samples were inactivated at 56 ℃ for 30 minutes. The test was performed by the constant virus-diluted serum method described previously (LU *et al.* 1990). The HmLu − 1 cell cultures and isolated virus (PT − 17 strain) were used for all serological studies. The antibody titer was expressed as the reciprocal of the highest serum dilution which could inhibit CPE of indicator virus.

RESULTS

Etiological Examination :

No significant bacteria was detected in tissues of necropsied calves.

Two strains of Akabane virus were isolated from blood liquid of the sacrificed calves (one from Taipei and one from Pintung districts), but none were from homogenate tissues of the sacrificed calves. The isolates caused prominent CPE in HmLu − 1 cells and could reach a high virus titer (5×10^6 $TCID_{50}$ per ml) which were subcultured in HmLu − 1 cells for 3 passges. The viral particles were round, oval or elongated and the diameter ranged from 90 to 100 nm under electronmicroscopy. A membrane-like envelope was occasionally observed (Fig. 1). These morphological features were compatible with those of Akabane virus as described by Ito *et al.* (1979).

Five strains of Akabane virus were isolated from blood samples of 11 cohabitating calves that had shown nervous signs in Pintung districts. Therefore, in addition to the afore-mentioned two strains isolated from sacrified calves, there was a total of seven strains of Akabane virus isolated from calves in the Pintung and Taipei districts. Furthermore, 30 blood samples of clinically healthy calves ranged from 4 to 12 months old were collected from six epizootic farms located in Tainan district (Table 1). Four strains of Akabane

virus were isolated from 3 of the 6 farms investigated.

Histopathological features of natural infection :

No gross lesion was found in necropsied calves.

The histopathological findings of the central nervous system (CNS) sere nonsuppurative encephalitis characterized by a moderate perivascular infiltration (Fig. 2), neuronal degeneration and neuronophages mainly affecting the brain stem and spinal cord. The skeletal muscle myofibers were discontinued or broken (Fig. 3), and there was a mild proliferation of satellite cells. Two calves had myofiber degeneration and focal atrophy.

Serological relationship between Akabane virus :

Since the morphological appearance of the isolates resembled the Akabane virus, the serological comparison of isolates was performed with the strains of Iriki, OBE − 1 and JaGar − 39 of Akabane virus. As demostrated by a cross neutralization test, the isolates had identical antigenicity to the Iriki strain of Akabane virus, but were antigenically more distant to the JaGar-39 and OBE − 1 strain of Akabane virus (Table 2).

Seroepizootical surveys :

All sick calves that died later or survived had antibody against the PT − 17 isolate. The antibody responses to the virus in endemic farms in Tainan district was 100 % positive against the isolate, PT − 17, in November of 1991 and the titers decreased gradually in the following May, June and July of 1992.

Table 1 Isolation of Akabane virus in epizootic districts in Taiwan, 1992.

District	No. of farm	No. of blood sample	No. of isolate
Tainan	6	30	4
Pintung	1	14	6
Taipei	1	1	1

Table 2 Cross neutralization test between the PT-17, Iriki, JaGar-39 and OBE-1 strains of Akabane virus.

Virus (strain)	Antibody titer against immune serum			
	PT-17	Iriki	JaGar-39	OBE-1
Taiwan (PT-17)	1,024	512	16	8
Akabane (Iriki)	1,024	1,024	8	4

Fig 1. The virus particles are round and its diameter varied from 90 to 100 nm. (Bar = 100 nm)

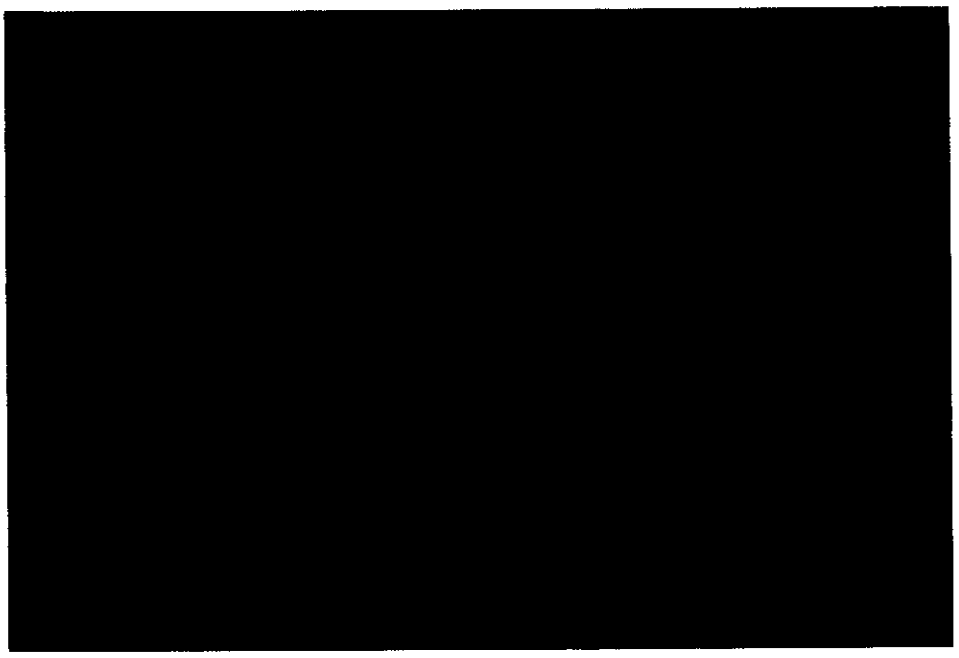

Fig. 2. The perivascular infiltration was observed in central nervous system in the sacrificed calves.

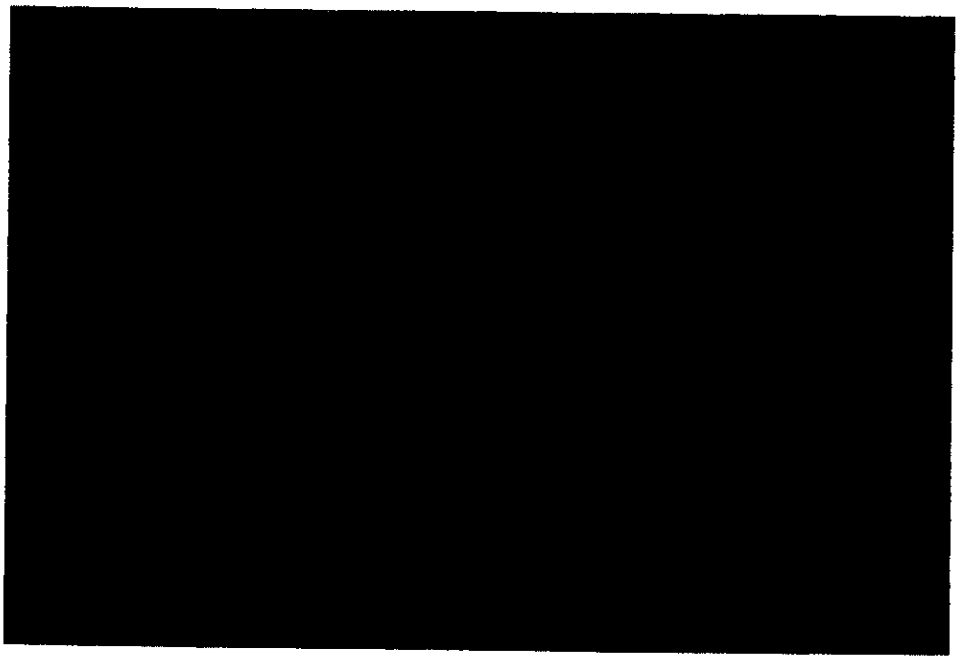

Fig. 3. The foci of skeletal muscles were observed discontinued or broken in sacrificed calves.

DISCUSSION

In the present study, Akabane virus was isolated from affected calves manifested with nervous signs and lameness. The isolate was considered to be Iriki virus group of Akabane virus by cross reaction with antiserum in virus neutralization test. The epizootiology of the present disease in Taiwan appeared to be similar to that noted in Japan where the Iriki virus was isolated from calves with encephalitis (MIYAZATO *et al.* 1989). Therefore, the Iriki virus isolated was considered to be the epizootical pathogen for the present occurrence. No virus was isolated from the CNS of necropsied calves in the present study, however, the virus has been successfully isolated in Japan from the brain of diseased calves by Miyazato *et al.* (1989). Furthermore, there were evidences of histopathologic effects on the CNS noted in clinical cases, we believed that the CNS lesions induced were related to the Iriki virus isolated. A further study on the pathogenesis of Iriki virus induced encephalitis is strongly recommented.

The Iriki viruses were also isolated from normal calf blood samples in epizootic farms of Tainan district. All cohabitated calves or calves with nervous signs that died later had high immune evidences against Iriki isolate. The seroconversion to the virus in epizootic farms which had prevalence of abortions or deformities was 100 % positive in November of 1991. Based on the serological data, we concluded that the Iriki virus had been widely transmitted among the endemic farms.

Arthrogryposis and hydraencephaly of bovine fetus has been seen as the main gross lesions of Akabane virus infection (INABA *et al.* 1990), and the arthrogryposis is believered to be resulted from the skeletal muscles and CNS damages in fetus, although the causes of arthrogryposis are often not clear (KUROGI *et al.* 1975, 1976). Akabane virus is known to induce slight and transient nervous signs in calves as described by Kurogi *et al.* (1987). There are some evidence on the virulence differances in the Akabane virus isolated, as Akabane virus has been isolated from normal bull (St. GEORGE *et al.* 1977) and sheep (DELLA – PORTA *et al.* 1977) in Australia. Nervertheless, the typical gross lesions of aborted fetuses with arthrogryposis and hydranencephaly caused by Akabane virus infected are less frequent in Japan in recent year (OIKE *et al.* 1988). In our study,

the virus was isolated not only from those affected calves but also from those calves without clinical symptome. These might indicate that there were differences in the virulence of isolates. We also suspected that these were possible divergences of Akabane virus in our epizootics. It will be interesting to conduct a further investigation on pathogenesis of the Iriki isolates in cows caused abortion. The Iriki isolates which had serological cross reactions with Akabane virus were noted in our present study. A further study aim at pathogenetic differences between the Iriki isolates and Akabane virus infection may be interesting as well.

The presence of antibody against low pathogenic Akabane virus had been noted in cattle in Taiwan (LU *et al.* 1990). It was believed that the presence of viruses and vectors probably induced a minimal immune reponse or protection to the cattle from ensuing disease. The cattle population had been fluctuated in Taiwan in recent years following frequent inducing new cows mostly from the States. These possibly resulted in non-immune situation to the Akabane virus among these cattle. And therefore, it could explain why there were only the occurrence of some sporadic abortions found in dairy farms, and why there were some affected calves with nervous signs. These Akabane virus affected calves probably lacked enough maternal antibody protection. For proper prevention and control, an effective vaccine against the Akabane virus is needed. The serological survey in sentinel cattle and possible migratory behaviors of vectors on endemic regions shall be studied also.

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在臺灣由仔牛分離赤羽病病毒 (Iriki strain) 的報告

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摘要 近年來在臺灣發現許多生後 4 至 12 月齡的仔牛發生非化膿性腦膜腦炎病例，以及酪農户中母牛發生流產或是畸型胎的疫情，爲探討病因而對本省牛隻受節肢動物媒介病毒 (Arbovirus) 感染與疾病的關係著手進行調查。結果於 1992 年的 6 月至 7 月期間在發生病例的疫區共分離出 11 株赤羽病病毒。這分離的病毒有 7 株是在 15 頭發生神經症狀的小牛血液中分離出。另外有 4 株則是在台南縣內曾經發生母牛流產及小牛異常產的 6 個乳牛場中臨床上看似健康的仔牛血液中分離出病毒。分離的病毒在 HmLu－1 細胞上會呈現明顯的細胞病變 (CPE) 現象，同時病毒力價可達 5×10^6 $TCID_{50}$ /ml。以交叉中和試驗方法而證明分離的病毒是爲赤羽病病毒。將臺灣分離毒株和日本分離赤羽病病毒株進行抗原性狀交叉中和試驗比較，結果本省分離株對日本 JaGAr－39 及 OBE－1 的免疫血清難以中和，而對日本分離的 Iriki strain 比較容易中和，因而由發生疫情及中和試驗的結果，證實臺灣分離株是屬 Iriki virus。這是本省首次在牛隻分離出赤羽病病毒的報告，也是繼日本之後分離出赤羽病病毒的 Iriki 株。

關鍵詞：赤羽病病毒，獸醫流行病學，入來病毒 (Iriki virus)

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