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DETECTION AND IDENTIFICATION OF RETROVIRUS ASSOCIATED WITH SHEEP PULMONARY ADENOMATOSIS IN MALAYSIA

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SUMMARY

The first detection and confirmation on the presence of jaagsiekte retrovirus (JSRV) is reported in sheep in Malaysia. The virus was detected by electron microscopy and was confirmed by examination of the nucleic acid using reverse-transcription polymerase chain reaction (RT-PCR). The JSRV was demonstrated exclusively and consistently in the lung fluid, lung tumour cells and the mediastinal lymph nodes of the suspected SPA-affected sheep

Keywords: Sheep pulmonary adenomatosis; retrovirus, Malaysia

INTRODUCTION

Sheep pulmonary adenomatosis (SPA), also known as as jaagsiekte and ovine pulmonary carcinoma, is a contagious disease of sheep that lead to lung cancer (DeMartini *et al.*, 1987; Temin 1988, Hecht *et al.*, 1994). Two viruses have been associated with this disease, a herpesvirus and a retrovirus (Sharp, 1991). Ovine herpesvirus, which was observed intranucleus (Sharp *et al.*, 1983; Zamri-Saad and Jasni, 1997) has been successfully isolated from tissues of sheep pulmonary adenomatosis (Sharp, 1991). Despite the strong associations of herpesvirus with sheep pulmonary adenomatosis, the virus is believed not to cause the disease especially when it was found unable to experimentally reproduce the disease (Sharp, 1991). Later, a jaagsiekte retrovirus was recognised as the major causative agent of sheep pulmonary adenomatosis. This virus was shown to transform the alveolar type II cells to become neoplastic cuboidal epithelial cells (Rosadio *et al.*, 1988).

The disease was first observed in Malaysia in 1993 involving mainly the pure-bred imported sheep and their crosses (Zamri-Saad *et al.*, 1996). However, only in 1994 that the first documented case of sheep pulmonary adenomatosis was recorded in Malaysia following histological confirmation of an outbreak of the disease (Krishnan and Paul, 1994). Subsequently, more cases of the disease were reported throughout Malaysia (Azman Shah *et al.*, 1996).

This report describes, for the first time in Malaysia, the detection of jaagsiekte retrovirus under electron microscopy and the subsequent identification of the virus using reverse-transcription polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Sources and preparation of samples

Three naturally SPA-affected sheep were used in this study. The affected animals showed typical clinical signs of sheep pulmonary adenomatosis, particularly the discharge of abundant sero-mucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head. Diagnosis of sheep pulmonary adenomatosis was made by gross and histological examinations of the lungs (Zamri-Saad *et al.*, 1996).

Sample collection and processing

Immediately after euthanasia, the lungs were collected and examined for gross lesions before they were washed by introducing 500mL of ice-cold sterile phosphate buffered saline (PBS) through the trachea while the lungs were massaged gently. The fluid was re-collect into a sterile container before another 500mL of the ice-cold PBS were introduced once again into the lungs, massaged and re-collected as previously described.

The freshly collected lung washing fluid was clarified by centrifugation at 11,000xg for 60 min at 4°C to remove the cellular debris. The fluid was then concentrated by further centrifugation at 100,000xg for 45 min at 4°C before the pellet was re-suspended in 0.05M phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 1 mM DTT. Large particles were disrupted in a homogeniser and the suspension was stored at -70°C until used.

Ten 10 grams of the affected lung tissue was collected and placed it in TNE buffer (0.01M Tris-HCl, 0.1M NaCl and 0.001M ethylenediamine-tetraacetic acid [EDTA] pH 7.4). The tissue was then homogenised in a Waring blender followed by centrifugation at 11,500xg for 30 min at 4°C to clarify the material. The supernatant was filtered (pore size 0.22μm) before it was subjected to ultracentrifuge at 98,000xg for 2 h at 5°C. The pellet was re-suspended in 5ml TNE buffer and kept at -70°C until further use (Rosadio *et al.*, 1988).

Virus purification

The stored suspension was thawed at room temperature before 2 volumes of cold fluorocarbon (Freon 113; Sigma) were added into the suspension and the mixture was shaken for 3 minutes. After phase separation using a Beckman JA 20 rotor (10 min at 10,000 rpm), the supernatant was collected while the inter-phase was extracted once more with TNE (0.1 M Tris hydrochloride (pH 7.5)-0.1M sodium chloride (NaCl)-0.001M EDTA). The combined aqueous phase was layered onto a 2.5mL layer of 30% sucrose TNE and centrifuged using a Beckman SW 41 rotor (1 h at 35,000rpm). The pellet was re-suspended in 1mL of TNE and further purified by layered onto a 20 to 50% sucrose gradient in a SW41 rotor (16 h at 25,000rpm).

Electron microscopic study

The positively and the negatively stained materials obtained from the purified procedure were prepared for transmission electron microscopy examinations according to the technique previously described by Payne *et al.* (1983). Thin sections of the purified materials were prepared before they were negatively stained with uranyl acetate.

RNA extraction

The virus RNA was extracted from the pooled sucrose gradient purified fractions using the acid guanidinium thiocyanat-phenol-chloroform method. Approximately 0.25 mL of the lung lavage fluid, 50 mg of the homogenised lung tumour tissue and 25 mg of the mediastinal lymph node were each added with 0.75 mL of TRIZOL LS Reagent (Life Technologies, USA) and mixed completely. The samples were incubated for 5 min at 15 to 30°C to permit complete dissociation of nucleoprotein complexes. Then 0.2 mL of chloroform was added into 0.75 mL of TRIZOL LS reagent before the tubes were shaken vigorously by hand for 15 seconds followed by incubation at 15 to 30°C for 2 to 15 min. The samples were then centrifuged at no more than 12,000xg for 15 min at 2 to 8°C. Centrifugation separated the mixture into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The RNA remained exclusively in the aqueous phase.

The aqueous phase was then transferred into a clean tube before the RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol at the rate of 0.5 mL of isopropyl alcohol per 0.75 mL of TRIZOL LS reagent. The mixture was then incubated at 15 to 30°C for 10 min and then centrifuged at no more than 12,000xg for 10 min at 2 to 8°C. The supernatant was carefully removed while the RNA pellet was washed once with 75% ethanol followed by an addition of 1 mL of 75% ethanol per 0.75 mL of TRIZOL LS reagent. The sample was then vortex-mixed and centrifuged at 7500xg for 5 min at 2 to 8°C. At the end of the procedure, the RNA pellets were dried briefly (air-dry or vacuum-dry for 5 to10 min) but not completely as this will greatly decrease its solubility. The RNA samples were then partially dissolved in RNase-free water by passing the solution a few times through a pipette tip followed by incubation for 10 min at 55 to 60°C.

Synthesis of cDNA

Synthesis of cDNA was carried out in 20 μL reaction volumes. Total RNA was diluted in 15 μL of diethyl pyrocarbonate treated distilled water. The RNA was denatured at 65°C for 10 min and then was cooled on ice. Five μL of the RNA of jaagsiekte retrovirus was mixed with 5 μL of distilled water, 1 μL of primer 2 and 1 μL of random primer before the mixture was heated to 70°C for 5 min and quickly chilled on ice for 2 min. The mixture was then spin before 4 μL of 5X first strand buffer, 2 μL of 0.1 M DTT and 1 μL of 10 mM dNTP mixture (each dATP, dGTP, dCTP and TTP at neutral pH) were added. The contents of the tube were mixed gently and incubated at 42°C for 2 min. One μL (200 units) of SUPERSCRIPT 11 was added into the tube, mixed properly by pipetting the content gently up and down, and incubated for 50 min at 42°C. Heating at 70°C for 15 min inactivated the reaction. The cDNA could then be used as a template for amplification in PCR.

Reverse-transcription polymerase chain reaction (RT-PCR)

The primers were designed based on the complete sequence of jaagsiekte retrovirus described by (York *et al.*, 1991) and the pCA1 sequence of gag (Nobel *et al.*, 1969). Primers P1 and P2 spanned a 229-bp region, internal to the gag gene that positioned between the 1,598 to 1,826 of jaagsiekte retrovirus (York *et al.*, 1991). Amplification of the resultant cDNA was carried out by polymerase chain reaction (PCR).

Detection of the polymerase chain reaction (PCR) product

The amplified PCR products were detected using electrophoresis. Twenty-five μL of the aliquots were passed through 2% agarose gel in 1x TBE buffer in the presence of 0.5 μg ethidium bromide per mL. Two μL of loading buffer were mixed with 5 μL of the PCR product as well as with the molecular weight marker (100bp DNA ladder). The pre-PCR, post PCR and negative control PCR sample mixtures were then loaded into the wells. Electrophoresis was carried out for 3 h at 100 V/2.5 mA. Following electrophoresis, the gel was washed three times with water and visualised under ultra-violet light transilluminator.

RESULTS

Electron microscopy

The virus particles resembling retrovirus were observed under the transmission electron microscopy. All the particles were observed in single form and rounded-shaped of approximately 70 to 100 nm in diameter (Fig. 1). The particles appeared double-walled with the inner ring clearer than the outer ring. The cores were small and condensed to form a rounded nucleoid. There was no evidence of projections on the surface of the viral particles.

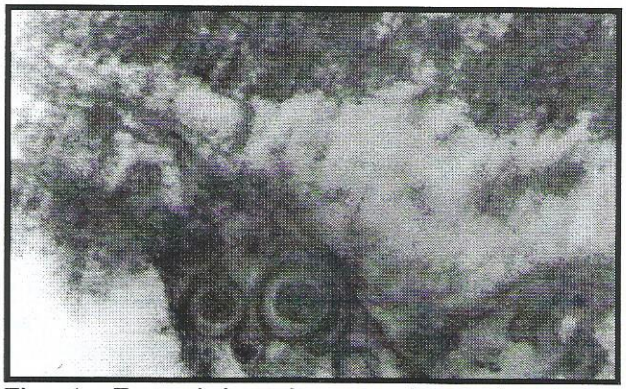

Fig. 1. Transmission electron micrograph of the jaagsiekte retrovirus observed in the lung fluid

Reverse-transcription polymerase chain reaction (RT-PCR)

Following RT-PCR, the minigel electrophoresis detection revealed the presence of 229 base pair band, which was considered positive for the RNA of jaagsiekte retrovirus (Fig. 2).

DISCUSSION

Two types of viral particles have been observed in the lungs of sheep with pulmonary adenomatosis; the herpesvirus and retrovirus (Sharp and Angus, 1990). The herpesvirus, which has also been reported in the nucleus of macrophages found in the lungs of sheep with naturally occurring sheep pulmonary adenomatosis in Malaysia (Zamri-Saad and Jasni, 1997), however, was not the causative agent of sheep pulmonary adenomatosis (Sharp and Angus, 1990, Sharp, 1991). This was mainly due to the main site of replication was the alveolar macrophages, not the tumour cells (Sharp and Angus, 1990) while the virus could not experimentally reproduced the disease (Sharp, 1991).

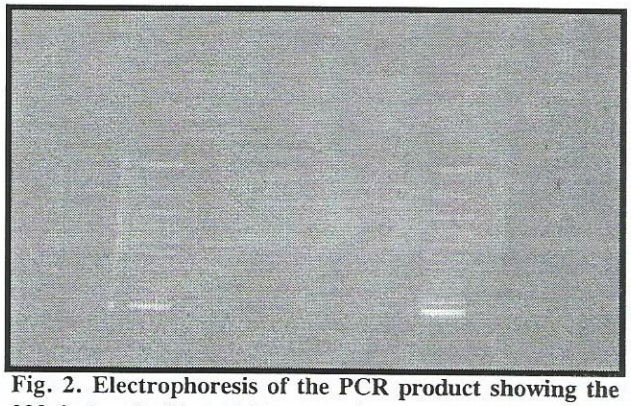

Fig. 2. Electrophoresis of the PCR product showing the 229 dp band of jaagsiekte retrovirus.

In the early 1970's, virus particles resembling retrovirus were detected in the sheep pulmonary adenomatosis tumour cells by electron microscopy (Sharp and Angus, 1990). Similarly, this study has reported detection of virus particles resembling retrovirus using the transmission electron microscopy. These particles resembling the jaagsiekte retrovirus reported by York *et al.* (1989). Although viral projections were reported following negatively staining procedure (Payne *et al.*, 1983), this study failed to identify the presence of viral projection. This, however, is in agreement with a later study by York *et al.*, (1989) who failed to observed any viral projections either with positively or negatively-stained materials.

Reverse-transcription polymerase chain reaction (RT-PCR) technique was employed to confirm the retrovirus. The technique produced a positive jaagsiekte sheep retrovirus (JSRV) band, considered to be the first detection and confirmation of the previously presumed JSRV infection in Malaysia. The RT-PCR was first used by Palmarini *et al.* (1995) to examine for the presence of JSRV gag transcripts in the respiratory and other tissues obtained from SPA-affected sheep. They found that the RT-PCR was a suitable technique to detect the JSRV in the suspected JSRV-infected sheep.

This study has provided compelling lines of evidence that JSRV and JSRV RNA were found in

the lung fluid, tumour cells and the draining lymph nodes of SPA-affected sheep. This is in agreement with the results of an earlier immunological study, which concluded that epithelial tumour cells are the major sites of replication for JSRV (Sharp and Angus, 1990). The lung fluid and tumour tissues of the SPA-affected sheep, however, are the only materials that have been used successfully to reproduce the tumour experimentally in sheep and goats (Sharp *et al.*, 1983; Sharp and Angus, 1990).

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RINGKASAN

PENGESANAN DAN PENGENALPASTIAN RETROVIRUS TERKAIT ADENOMATOSIS PULMONARI BIRI-BIRI DI MALAYSIA

Pengesanan dan pengesahan pertama kepada wujudnya retrovirus jagsiekte (JSRV) dilaporkan pada biri-biri di Malaysia. Virus dikesan melalui mikroskopi elektron dan disahkan melalui pemeriksaan asid nukleik mengguna tindak balas rangkaian polimerase pentranskripsian berbalik (RT-PCR). Penunjukan JSRV ini berlaku secara eksklusif dan berterusan dalam bendalir paru-paru, sel tumor paru-paru dan nodus limfa mediastinum pada biri-biri terkesan adenomatosis pulmonari biri.