Studies on bluetongue disease in the People's Republic of China

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Summary

Bluetongue (BT) is an important infectious, non-contagious, insect-borne viral disease of ruminants, and is classified as a 'List A' disease in the OIE *Terrestrial animal health code*. Since the first discovery and diagnosis of this disease in the Shizong County of Yunnan Province in 1979, the authors have developed systematic studies of the epidemiology, experimental epidemiology, aetiology, pathology, viral molecular characteristics (nucleic acid), diagnostic techniques, virus identification (grouping and typing) methods, vaccines and immunisation methods of BT in the People's Republic of China. Viruses have been isolated from seven provinces and the major serotypes have been identified. Epidemiological surveys have been conducted in sixteen provinces and a distinct type of BT virus (BTV) epidemiology has been found in China. Sentinel herds were established to monitor BTV regularly from 1995 to 1998. Experimental epidemiological studies have revealed the regional distribution of BTV serotypes and the dynamic spread of BTV in different habitats of distinct natural conditions, ecological environment and climate in China. A technical system of diagnosis of BTV infection also has been developed, including the agar gel immunodiffusion (AGID), indirect ELISA, competitive-ELISA (c-ELISA) and virus neutralisation (VN) tests for detection of antibodies to BTV, and the serum neutralisation (SN), immunofluorescence (IF) and immunoperoxidase (IP), antigen capture-ELISA (ac-ELISA), virus inoculation methods for virus detection, and nucleic acid electrophoresis analysis, nucleic acid probe techniques, and polymerase chain reaction (PCR) methods for detection of BTV nucleic acid and/or proteins. Attenuated and killed vaccines of BTV serotypes 1 and 16 have been developed and new immunisation methods have been developed. The S7, S10 and portions of the L2 gene segment of Chinese prototype strains of BTV serotypes 1, 2, 3, 4, 12, 15 and 16 have been sequenced and compared to the same strains of prototype and field strains of BTV from the United States of America and Australia. Phylogenetic analyses indicate that the genetic relationships between these viruses correspond to their geographic origins or their serotype. The National Standards for Bluetongue Diagnosis, People's Republic of China has also been enacted. Remarkable social and economic benefits have been obtained through the application and dissemination of these achievements in China.

This paper reviews the progress of studies and control of BT in China since 1995.

Keywords

Bluetongue virus – Diagnosis – Epidemiology – People's Republic of China – Vaccines.

Epidemiology of bluetongue in China

Epidemiological survey

Bluetongue (BT) normally occurs only in sheep, and fine-wooled breeds of sheep (like the Merino) are more sensitive. Cattle, and possibly buffalo, are the main vertebrate reservoirs of the virus (2). BT was first recognised in China in 1979 in Yunnan Province, and both the disease and antibody have since been recorded in other provinces in China (7, 12, 22, 23).

A total of 224 000 animals of nine different species from 29 provinces were surveyed to investigate the epidemiology of BT in China. Four species of ruminants (goat, sheep, buffalo, cattle) were found to be susceptible (20), and the presence of four vector species of *Culicoides*, including *C. actoni*, was confirmed (10). Camels were not affected. The

epidemic period of BT coincides with the peak activity of *Culicoides* (from June to October), and there is an obvious seasonal cycle. Epidemics of clinical BT in sheep occurred in the region south of 35°N to 37°N, whereas only seroconversion occurred in ruminants in the regions north of 35°N to 37°N, such as Inner Mongolia where goats showed higher BT virus (BTV) group antibody prevalences than cattle, in contrast to the findings in most other regions. Another interesting result in Inner Mongolia is that some BTV infections continue in the winter months, suggesting an additional vector system; this finding contrasts with those in subtropical regions and other temperate areas of the world (22, 23).

Epidemiological survey of serotype-specific antibodies

For the serological investigation of BT in China, a total of 7 158 serum samples were collected from goats, sheep, buffalo and cattle in seven provinces (Yunnan, Shanxu, Sichuan, Hubei, Inner Mongolia, Xinjiang and Tibet) from 1994 to 1997. A total of 1 256 serum samples that were positive by AGID or c-ELISA were tested in micro-SN tests using international reference strains of BTV serotypes 1, 2, 3, 4, 5, 9, 15, 16, 17, 20, 21 and 23, the serotypes that are known to be present in Asia and the Pacific. Antibodies to seven serotypes, namely 1, 2, 4, 9, 15, 16 and 23 were found. In China, the dominant antibodies are to serotypes 1, 2, 4 and 16. The results further suggested that BTV-4 might pose the greatest threat in China. Antibodies were also detected against two or three serotypes of BTV in single serum samples, indicating that cross-reactions occur amongst the serotypes of BTV that are present in China. The results were similar to the results of virus isolation studies, and the survey for BTV antibodies will assist in the epidemiological and pathological investigations of BT in China (9, 21).

Epidemiological study of bluetongue virus in China

Six sentinel herds of cattle, buffalo or goats have been established in different regions of Yunnan Province that have different climates, natural ecosystems and environment, to study the epidemiology of BTV infection (11). To determine the geographical distribution of BTV and to ascertain the range of serotypes that may be present in China, serum and whole (unclotted) blood samples were regularly collected from sentinel animals to monitor virus activity and to isolate the virus. A total of 110 isolates of BTV were obtained from sentinel cattle, and these were identified by virus and serum neutralisation tests (VN and SN). Serotypes isolated included 1, 2, 3, 4, 12, 15 and 16. Except for BTV serotypes 1 and 16 that have been isolated from sheep with clinical disease or naturally infected goats, the other serotypes (2, 3, 4, 12 and 15) had not been isolated previously in China. BTV serotypes 3, 4, 5 were obtained in Shiaong (which had outbreaks of BT in 1979) and BTV serotypes 1, 3, 4, 15 and 16 in Eshan in the subtropical area of Yunnan Province. The highest level of transmission took place in July and August, which coincided with BT outbreaks in Yunnan. BTV serotypes 1, 2, 12 and 16 were obtained in Xishuangbannai (Jinhong) and BTV serotypes 4, 12 and 15 in Dehong in the tropical region of Yunnan Province, although the date of the peak of transmission is not clear (8, 26). The presence of the vector in this region is extended because of the climate and environment, thus seroconversion of animals occurs throughout the year in Xishuangbannai (Jinhong) and Dehong. In contrast, neither seroconversion of animals nor virus isolation was detected in the 15 goats and 2 cattle in the Kunming sentinel herd. The seropositive cattle as well as those from which BTV was isolated had no obvious clinical signs of BT during the monitoring period, suggesting that cattle provide a virus reservoir in the epidemiology of BTV infection (2). Together, these data show that sentinel herds can provide valuable data on the distribution of BTV serotypes and transmission of the virus. This information will ultimately be useful for the prevention and control of BT (8, 26).

Sequence comparison of the L2, S7 and S10 genes of bluetongue viruses from the People's Republic of China and other countries

The S10 and a portion of the L2 gene segments of Chinese prototype strains of BTV serotypes 1, 2, 3, 4, 12, 15 and 16 were sequenced and compared to the same genes of prototype and field strains of BTV from the United States of America (USA). Phylogenetic analysis of the S10 gene segregated the Chinese viruses into a monophyletic group distinct from the USA viruses, whereas similar analysis of the L2 gene segregated strains of BTV according to serotype, regardless of geographic origin. Phylogenetic analyses of these viruses also indicate that they are more closely related to one another, and to an Australian strain of serotype 1, than they are to prototype strains of bluetongue virus serotypes 2, 10, 11, 13 and 17 from the USA. The S7 gene segments of these viruses were sequenced and compared to the same genes of prototype strains of BTV from the USA, Australia and South Africa. The S7 genes and predicted VP7 proteins of the Chinese viruses were relatively conserved, with the notable exception of

serotype 15. Furthermore, phylogenetic analysis of the S7 genes did not predict geographic origin of the various strains of BTV (3, 4, 27).

Study and establishment of laboratory systems for diagnosis and monitoring of bluetongue in China

The diagnosis and monitoring of BT have been studied and established using the agar gel immunodiffusion (AGID) test, indirect ELISA, c-ELISA and VN tests for detection of antibodies to BTV in ruminants, and the SN, immunofluorescence (IF) and immunoperoxidase (IP) methods, antigen capture-ELISA (ac-ELISA), polymerase chain reaction (PCR) and virus inoculation methods for the isolation and identification of BTV.

Techniques for monitoring the presence of bluetongue virus

A system was developed for the isolation of BTV, namely: whole blood from ruminants is inoculated into chicken embryos that are then tested by ac-ELISA, blind passage in C6/36 cells, adaptation of isolated viruses to BHK-21 and Vero cells and, finally, virus identification. Red blood cells from whole (anti-coagulated) blood samples are disrupted and diluted prior to intravenous inoculation into chicken embryos. After incubation, liver samples are collected, homogenised, centrifuged, the supernatant collected and tested by ac-ELISA. Only positive samples to the ac-ELISA are passaged in cell culture to isolate viruses (firstly with one blind passage in C6/36 cells, followed by up to three passages in BHK-21 cells). By using this method, all serotypes of BTV were recovered from blood samples collected from experimentally inoculated sheep. A total of 110 isolates of BTV were obtained from 1 348 blood samples collected from sentinel cattle. The results show that this is a rapid, accurate and successful system for the isolation of BTV, and that it is especially useful in identifying subclinical BTV infection of cattle (8).

Bluetongue antigen capture enzyme linked immunosorbent assay

The BTV ac-ELISA was used and refined with polyclonal and monoclonal antibodies to detect BTV serogroup antigens. The assay was used to test samples that had been collected from inoculated sheep and the results were compared with traditional virus isolation methods. Identical results were obtained. Tests were conducted on 2 745 chicken embryo samples (inoculated with 495 clinical blood samples) and revealed 450 embryo samples (inoculated with 96 blood samples) to be positive to the ac-ELISA. From 96 blood samples, 83 isolates of BTV were obtained, confirming that 86.5% of results were identical between the two assays. The results indicated that the ac-ELISA is an optimal method for detection or identification of BTV serogroup antigen in chicken embryos and cell culture fluid, and that use of ac-ELISA can shorten the period and improve efficiency of virus isolation and identification (6, 8).

Bluetongue immunoperoxidase staining test

The BTV IP staining test was used with serogroup-specific monoclonal antibodies (MAb) for detection and identification of BTV serogroup antigens. The 24 international reference strains of BTV and related viruses (epizootic haemorrhagic disease virus, etc.) were used to evaluate the specificity of the IP test. The results showed this method can detect all 24 serotypes of BTV, without cross-reactions with related viruses. All 110 BTV isolates from sentinel cattle in China were identified using the IP test, and the results were identical to those obtained with the traditional BTV IF antibody test. This simple method provides a sensitive and specific assay for detection of BTV serogroup antigens (8).

Detection of bluetongue virus by polymerase chain reaction

The PCR assay was used to confirm the presence of BTV in tissue culture, chicken embryos, and clinical blood samples. BTV VP3-specific oligonucleotide primers were used in PCR-based diagnostic tests. The VP3 oligonucleotide primers that successfully amplified gene sequences from the BTV isolates from China previously were successfully tested on BTV isolates from many temperate regions around the world. A set of oligonucleotide primers with sequences derived from the Australian BTV serotype 1 VP2 sequence was tested for its ability to amplify VP2 sequences, irrespective of viral serotype. From an analysis of these VP2 PCR reactions, a minimum of three separate serotypes were present in the isolates tested. A PCR analysis of blood samples from Shanxi, performed in 36 hours, demonstrated the speed and efficiency with which a positive diagnosis could be made, and compared very favourably with the traditional diagnostic and tissue culture methods for identifying BTV in ruminant blood (15, 16).

Bluetongue competitive enzyme-linked immunosorbent assay

The AGID and c-ELISA (which incorporates BTV serogroup-specific MAbs 8A3B6 and 7D3A2) (1) were used to test 560 serum samples that were collected from goats, sheep and cattle in seven provinces in China. The results obtained with

414 samples were identical. A total of 75 sera that were positive in the c-ELISA were negative in the AGID, while seven samples positive in the AGID were negative in the c-ELISA. The results suggest that the c-ELISA may be more sensitive and specific than AGID in the detection of antibodies to BTV. In tests of 5 087 serum samples from ruminants, 1 679 samples were positive using c-ELISA (% inhibition >40%). The results showed that the c-ELISA is an optimal method for serological surveys of BTV infection.

Identification of local isolates of bluetongue virus in China

Storage and preparation of bluetongue reference viruses and antiserum

International reference strains of BTV of all 24 serotypes (originally obtained from the World Reference Centre, Onderstepoort, South Africa) and Chinese reference strains of BTV (isolated and identified by the Tropical and Subtropical Animal Virology Laboratory in China) were passaged and multiplied in BHK-21 cells, then adapted to Vero cells and titres determined. Reference antiserum was produced for each virus by inoculation of susceptible animals (healthy sheep). These studies showed that:

a) antibody and virus were detected at different intervals after infection of animals with the various viruses, and that the neutralising antibody titres of individual antisera differ
b) using virus-infected BHK-21 cell supernatant fluid as the inoculum, antisera may exhibit toxicity for BHK-21 cells but not for Vero cells, indicating that the cell lines used in the preparation of antiserum and in the neutralisation test must be different and that strains of BTVs used in the neutralisation test should be adapted to Vero cells
c) the neutralising antibody titre of antiserum produced was higher than the international reference antiserum from South Africa.

The storage system for reference viruses is a model for the storage of other viruses and bacteria.

Identification of local strains of bluetongue virus

Since the first outbreak of BT in Yunnan in 1979, BTVs have been isolated from sheep in Yunnan, Hubei, Sichuan, Anhui, Shandong and Shanxi Provinces, from goats in Xinjian and Inner Mongolia, and from cattle in Gansu. These virus strains were identified as BTV by AGID, IF and agar gel electrophoresis. Eight of the Chinese strains were serotyped by micro-SN tests. These showed that strains S×1 from Yuncheng County in Shanxi, S×2 from Jiansu County in Shanxi, Y863P12 from Yunnan, ×27 from Xinjian and Yc from *Culicoides* trapped in the animal laboratory in Kunming were all BTV serotype 1. Both WP7 from Hubei and SWP7 from Sichuan Province were BTV serotype 16. Early results indicate that NMP11 from Inner Mongolia might be BTV-17 but further confirmatory tests are required. In 1998, 110 strains of BTV were isolated from subclinically infected cattle in four sentinel herds in the Yunnan Province. Using the international reference antiserum, the BTV strains were identified to serotypes using the micro-neutralisation test. The results showed:

a) the serotypes isolated included 1, 2, 3, 4, 12, 15 and 16
b) the predominant serotypes were 1, 4, 15 and 16
c) during short periods of just one or two months, two or three different BTV serotypes were isolated from the same cattle. This suggests that animals were either infected with more than one BTV serotype, or that they can be infected sequentially with multiple serotypes
d) these results have provided considerable insight into the distribution and seasonal transmission of BTV in China (8, 25, 26, 27).

Detection and identification of bluetongue serogroups and serotypes by reverse transcriptase-polymerase chain reaction

Based on the sequence of BTV RNA segments L2 and L3, sets of oligodeoxynucleotide primers were designed and synthesised to establish the BTV serogroup- and serotype-specific reverse transcriptase-polymerase chain reaction (RT-PCR) assays. The serogroup-specific RT-PCR assay can detect all Chinese BTV serotypes, without cross-reactions with other related viruses. The serotype-specific RT-PCR assays for BTV serotypes 1 and 16 detect only the appropriate 'self-serotype' and no cross-reaction occurs with other serotypes. This RT-PCR technique was applied to identify clinical isolates and detect BTV in clinical samples (red blood cells and tissue samples) from inoculated sheep. The results were identical to the traditional methods. The results also indicated it is a simple, highly sensitive and specific method for the detection and identification of BTV.

Bluetongue vaccines

Development of bluetongue vaccines

Chicken embryo-adapted attenuated and inactivated vaccines of BTV serotypes 1 and 16 were successfully developed and produced. The protection

rate of attenuated vaccines and inactivated vaccines were greater than 90% and 75% respectively, and gave immunity that lasted one year and more than six months, respectively. The technical procedure of viral nucleic acid inactivation has been implemented. A total of 220 000 animals from Yunnan, Sichuan, Hubei, Shanxi and Jiangsu Provinces were vaccinated, which has controlled the incidence of clinical BT disease in China.

The effective vaccination schemes were implemented using single or combined vaccination in different epidemic areas (5, 12, 13, 17, 18, 19, 24, 28).

Immunomorphology of vaccinated sheep

No viral antigen was detected in the target cells of vaccinated sheep by immunohistochemistry, histology, cellular chemistry and electron microscopy. The immune response to BTV infection was both humoral and cellular. In the immune system, T and B cells and peripheral blood peaked at the same time. No viral antigen was detected in the target cells of vaccinated sheep after challenge (24).

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