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Seroprevalence of five bovine arboviruses and development of inactivated trivalent vaccine for Aino, Akabane and Chuzan viruses in Korea

소 아보바이러스 5종의 유병률 및 아이노, 아까바네, 츄잔바이러스 3종 불활화 혼합백신 개발

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By

Yeon-Hee Kim

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Major in Veterinary Pathobiology and Preventive Medicine

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Abstract

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Arthropod-borne viruses (arboviruses) are transmitted by blood-sucking arthropods, such as mosquitoes, *Culicoides* biting midges and ticks. These arbovirus infections are mainly associated with abortion, stillbirth and congenital defects in pregnant cattle, sheep and goats, which induces a considerable economic loss in livestock industry.

To investigate the possible circulation of arboviruses in South Korea, nationwide surveillance of five arbovirues was conducted in sentinel calves during 2009−2012. We used serum neutralization tests to investigate the presence of antibodies for the Aino virus, Akabane virus, bovine ephemeral fever virus, Chuzan virus and Ibaraki virus. In 2009, 2011 and 2012, the seropositive rates for these five arboviruses were all less than 14.1%. In 2010, however, the seropositive rates for Aino virus and Akabane virus were 33.2% and 40.2%, respectively. High seropositive rates were also associated with a large-scale outbreak of Akabane viral encephalomyelitis in cattle in southern Korea in 2010. Continued seroprevalence

surveillance will be useful for monitoring natural arboviral diseases.

The arboviruses seem to be widely distributed in Southeast Asia and Australia. Among the arboviruses, Aino, Akabane and Chuzan viruses are respectively isolated in abortion cases. As a control strategy, an inactivated trivalent vaccine against Aino, Akabane and Chuzan virus was developed by using binary ethylenimine or formalin as an inactivating agent. The newly developed trivalent vaccine is evaluated for its safety and immunogenicity in animals such as mice, guinea pigs and cattle. The immune responses were significantly detected within 2-weeks after second vaccination without any side effects. Since the field application of experimental vaccine also revealed increased antibodies in inoculated cattle, we demonstrated that these trivalent vaccines could be used as a vaccine to control the arboviral infections in ruminants.

This study approached the seroprevalence of five arboviruses in sentinel cattle and development for prevention of the diseases on Aino, Akabane and Chuzan viruses in cattle.

Key words: arbovirus, seroprevalence, aino, akabane, chuzan virus, trivalent vaccine

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Abbreviations

ACRONYM	FULL NAME
Arboviruses	Arthropod-borne viruses
AINOV	Aino Virus
AKAV	Akabane Virus
BEFV	Bovine Ephemeral Fever Virus
CHUV	Chuzan Virus
IBAV	Ibaraki Virus
KVCC	Korea Veterinary Culture Collection
SNT	Serum Neutralization Test
CPE	Cytopathic effects
$TCID_{50}$	Tissue Culture Infective Dose
HmLu	Hamster lung
BHK	Baby hamster kidney
BEI	Binary Ethylenimine
MOI	Multiplicity of Infection
SPF	Specific Pathogen-Free
SC	Subcutaneous
IP	Intraperitoneal
IM	Intramuscular
JE	Japanese encephalitis

General Introduction

Epizootic congenital abnormalities, encephalomyelitis and febrile illnesses in cattle caused by arthropod-borne viruses (arboviruses) are prevalent in South Korea. Causative viruses including orthobunyaviruses, bovine ephemeral fever virus, Chuzan virus, Ibarakivrus are thought to be transmitted by *Culicoides* biting midges. Isolation and antibody surveys of arboviruses have previously been reported in Australia, Asia, Africa and the Middle East (Jagoe et al., 1993; Jun et al., 2012, Lim et al., 2007; Metselaar et al., 1976; Taylor et al., 1994; Yeruham et al., 2010).

In this study, serological monitoring of five arboviruses was conducted during 2009-2012 using a total of 4,000 blood samples, with samples collected from 500 unvaccinated sentinel cattle twice each year. The study was carried out in all provinces and metropolitan cities of the Republic of Korea. In May and October of each year from 2009 through 2012, blood samples were collected from 500 unvaccinated Korea calves, yielding 1,000 blood samples annually. Seropositive animals were those that were antibody negative in May, but antibody-positive in October of the same year. Therefore, the seroprevalences of the five viruses in this study represent the proportions of the 500 sampled in dividuals that tested positive for the antibody in each year. Collecting serum samples from the same individuals before and after the vector season provided reliable epidemiological data for arboviral infection.

Any increase in temperature results in an increase in the range, abundance and seasonal activity of *Culicoides* biting midges, which are capable of transmitting the

bovine arboviruses (Yanase et al., 2005; Purse et al., 2005). In Japan, a monitoring in *Culicoides* biting midges and sentinel cattle detected the circulation of Akabane virus just prior to the accumulation of bovine congenital abnormalities and encephalomyelitis by it around study sites in 2003, 2006, 2008 and 2013 (Kato et al., 2015). Contiued seroprevalence surveillance and mornitoing of vectors will be useful for alarming natural arboviral disease. Given the current economic importance of these viral infections, and the prospect that the geographic range of the biting midge will increase by global warming, various protective measures including vaccination have been recognized (Forman et al., 2008; Tsutsui et al., 2009).

In Japan, a multivalent vaccine together with AKAV, AINOV and CHUV has developed for disease control and is available in the field (Kurogi et al., 1978; Hechinger et al., 2013).

Akabane vaccines have been commercially available in Korea since 1990. However, it is reported that positive rates against other arboviruse diseases have also been increasing (Lim et al., 2007; Yang et al., 2008). For protecting not only Akabane virus but also other arboviral-mediated diseases, the development of multivalent vaccine is required in the field circumstances. As an approach, an inactivated trivalent vaccine against an Aino, Akabane and Chuzan virus was developed and evaluated.

General Methodology

A. Cell culture virus isolation

i) Virus isolation can be conducted in cell lines and primary cells susceptible to arbovirus infection. Vero cell, BHK-21, HmLu cell line is the preferred cell line.

ii) Wash confluent cell monolayers (24-48 hours post seeding) three times with cell culture medium. The cell culture medium can be supplemented with antibiotics, but is not supplemented with fetal bovine serum.

iii) Inoculate cell cultures with an appropriate amount of tissue suspension. Note: The volume of inoculums will vary with the size of the cell culture container. In general, 100-200 ul are inoculated in each well of a 24-well culture plate, 1-2 ml into a 25 cm^2 flask.

iv) Incubate inoculated cell cultures for 1-2 hours at 37℃.

v) Remove the inoculums and wash the cell monolayer three times with the cell culture medium.

vi) Add an appropriate volume of the cell culture maintenance medium to all containers and incubate at 37℃ for 5-7 days with periodic examination for cytopathic effect (CPE). If CPE is observed, an aliquot of the cell culture medium can be tested by reverse transcription-polymerase chain reaction (RT-PCR) for conserved virus genes.

B. Serum neutralization

SN tests have been described using HmLu-1 cells, Vero or BHK cells in flat-bottomed 96-well microtitre plates (Da Costa Mendes, 1984; Weir, 2003). In case of AKAV, two techniques have been described with a serum/virus incubation period of 1 hous or incubation overnight before the addition of the cells.

i) Inactivate the test sera at 56℃ for 30 minutes in a water bath.

ii) Prepare serial twofold dilutions of the sera in Eagles medium form 1/2 to 1/16 in a 96-well flat bottomed microtitre plate using duplicate wells and 25 ul per well. Standard controls are prepared in a similar way.

iii) Add 25 ul per well of virus in Eagles medium diluted to provide 200 $TCID_{50}$ per 50 ul.

iv) Cover and incubate at room temperature for 1 hour.

v) Include a back titration of virus in triplicate, making three tenfold dilutions using 25 ul per well.

vi) Add 100 ul per well Vero cells in Eagles medium with 2% serum at 5 x 10^5 cells/ml

vii) Incubate the plates at 34-37℃ for 5 days in a humidified CO_2 incubator.

viii) Read the plates microscopically and calculate the titre as the reciprocal of the highest serum dilution completely inhibiting the CPE.

ix) The virus and serum controls should give the expected results.

Where overnight incubation is used, duplicate twofold serial dilutions of inactivated serum are mixed with 100 $TCID_{50}$ of virus using 100 ul volumes in each case. Following incubation for 1 hour at 37℃ and

overnight at 4℃, 50 ul BHK cells is added to the test. The plate is examined at 3 and 5 days incubation at 37 ℃ and checked for CPE.

C. Plaque assay

The plaque assay is the most quantitative and most useful biological assay for viruses. The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells.

The below procedure applies to perfrom a plaque assay in 6-well plate format to determine the titer.

i) On the day of infection, harvest the Vero cells and prepare a 30 ml cell suspension at 5 x 10^5 cells/mL in cell culture medium. Aliquot 2 mL of cell suspension into each well of two 6-well plates. If you are including a negative control, you will need another 6-well plate.

ii) Allow the cells to settle to the bottom of the plate and incubate, covered, at room temperature for 1-2 hour.

iii) Following the incubation, observe the cell monolayers using an inverted microscope. Cells should be attached and at 50 % confluence.

iv) Prepare an 8-log serial dilution (10^{-1} to 10^{-8}) of the virus in cell culture medium, supplement without FBS, as appropriate. To do this, sequentially dilute 0.5 mL of the virus or previous dilution in 4.5 mL of medium in 12 mL disposable tubes.

v) Move the 6-well plates containing cells and the tubes of diluted virus to the sterile hood. Label the plates,as follows: no virus (negative control),

10^{-2}, 10^{-3}.

vi) Remove the medium from each well, discard and immediately replace with 1 mL of the appropriate virus dilution. As a negative control, add the appropriate medium without virus.

vii) Incubate cells with virus for 1 hour at room temperature. Following the 1 hour incubation, move the cells and the bottle of plaquing medium from the 40℃ water bath to a sterile hood.

viii) Sequentially, starting from the highest dilution to the lowest dilution, remove the medium containing virus from the wells and replace with 2 mL of plaquing medium. Work quickly to avoid dessication of the cell monolayer.

ix) Allow agarose overlay to harden for 1 hour at room temperature before moving the plates.

x) Incubate the cells in a 27℃ humidified incubator for 7-10 days until plaques are visible and ready to count. If you wish to stain plaques to facilitate counting, prepare a 1 mg/mL Neutral Red solution in cell-culture grade, distilled water.

xi) Add 0.5 mL of Neutral Red solution to each well containing plaquing overlay. Incubate for 1 to 2 hours at room temperature.

xii) Gently remove excess stain with a pipet or blotter and count the plaques. Plaques will appear as clear spots in a nearly clear gel against a red background.

Count the number of plaques present in each dilution, then use the following formula to calculate the titer (plaque forming units (pfu)/mL)

of your virus. Note that the optimal range to count is 3 to 20 plaques per well of a 6-well plate.

Titer (pfu/mL) = number of plaques x dilution factor x 1/ mL of inoculums per well

In this example, we add 1 mL of inoculums and observe 20 plaques in the well containing the 10^{-6} viral dilution. Using the formula above, the titer of this viral stock is : titer (pfu/mL) = 20 plaques x 10^{6} x 1/1mL per well = 2 x 10^{7} pfu/mL

D. Multiplicity of infection (MOI)

Multiplicity of infection, often abbreviated "MOI", measures the average amount of virus added per cell in an infection. MOI can be expressed using any quantitave measure of virus titer, for example: particles/cell, HA units/cell, $TCID_{50}$/cell, or pfu/cell. To amplify your viral stock, infect cells at a MOI ranging from 0.05 to 0.1 MOI is defined as the number of virus particles per cell.Use the following formula to calculate how much viral stock to add to obtain a specific MOI:

Inoculum required (mL) = MOI (pfu/cell) x number of cells/ titer of viral stock (pfu/ml)

E. Principles of veterinary vaccine production

The nomenclature for veterinary biological products varies from country to country. According to manual of diagnostic tests and vaccines for terrestrial animal, principles of veterinary vaccine production and the term are

introduced.

1. Vaccine types

Vaccines may be prepared as live or inactivated (killed) products. Some live vaccines are prepared from low virulence, mild, field isolates of a disease-causing agent that have been found to be safe and effective when administered. Other live vaccines are prepared from isolates of disease-causing agents that have been modified by passage through laboratory animal, culture media to select a variant of reduced virulence.

Killed products may contain: 1) cultures of microorganisms that have been inactivated by chemical or other means; 2) inactivated toxins; 3) subunit (antigenic parts of microorganisms) that have been extracted from cultures or that have been produced through rDNA procedures.

Both live and inactivated vaccines may be formulated with adjuvants designed to enhance their efficacy. Frequently used adjuvants are typically water-in-oil emulsions (either single or double), made with mineral or vegetable oil and an emulsifying agent. Other adjuvants, such as aluminium hydroxide gel or saponin, are also used.

2. Requirement for vaccines

1) Master seed

The objective of testing the master seed is to ensure vaccine safety, quality and efficacy. Safety should be tested in an early stage. A master seed should be established for each microorgamism used in the production of a product to serve as the source of seed for inoculation of all production cultures.

Working seeds and production seeds may be prepared from the master seed by subculturing. The master seed should be frozen or desiccated and stored at low temperatures such as -70℃ or under other conditions found to be optimal for maintaining viability. Each master seed should be tested to ensure its identity, safety and efficacy.

2) Method of culture

Arboviruses seed can be grown in cell culture, such as Vero, BHK cells. Selection of culture method is dependent on the degree of virus adaptation, growth in medium, rate of mutation and viral yield in the specific culture system.

The purity of the seed and cells to be used for vaccine production must be demonstrated. The master seed virus should be free from adventitious agents, mycoplasma or extraneous virus, using tests known to be sensitive for detection of these microorganisms. Cultures are subpassaged at 3-5day intervals for a total of at least 7 days, then tested for cytopathogenic. Cells are also examined for adventitious viruses that may have infected the cells or seed during previous passages. Potential contaminants include bovine viral diarrhea virus, reovirus, rabies virus. Cell lines on which the seed is tested include: an African green monkey kidney (Vero) cell line, a cell line of the species of cells used to propagate the seed and cell lines for any other species through which the seed has been passaged. Bovine viral diarrhea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

3) Validation as a vaccine

Vaccine candidates should be shown to be pure, safe, potent and efficacious. Purity is determined by testing for a variety of contaminants. Tests to detect contaminants are performed on: master seed, primary cells and each batch/serial of final product prior to release. Purity test procedures have been published, for example in the European Pharmacopoeia, in CFR title 9 part 113, for the detection of extraneous viruses, bacteria, mycoplasma and fungi agents and pathogens detected by enzyme-linked immunosorbent assay, polymerase chain reaction, or the fluorescent antibody technique. Procedures used to ensure that fetal or calf serum and other ingredients of bovine origin are free of pestiviruses should be high concern and well documented.

Safety tests are required for the releases of each batch/serial. Standard procedures are given for safety tests in mice, quinea-pigs, sheep, cats, dogs and are generally conducted using fewer animals than are used in the safety tests required for licensing. Batches/serials are considered satisfactory if local and systemic reactions to vaccination with the batch/serial to be released are in line with those described in the registration dossier and product literature. Some authorites do not permit batch/serial safety testing in laboratory animals, requiring a test in one of the target species for the product.

Potency tests, required for each batch/serial prior to release, are designed to correlate with the host animal vaccination-challenge efficacy studies. For inactivated viral or bacterial products, potency tests may be conducted in

laboratory or host animals. The potency of live vaccines is generally measured by means of bacterial counts or virus titration. When testing a live viral vaccine for release, the virus titre must, as a rule, be sufficiently greater than that shown to be protective in the master seed immunogenicity test in order to ensure that at any time prior to the expiry date, the titre will be at least equal to that used in the immunogenicity test. It is evident that the appropriate release titre is primarily dependent on the required potency and secondarily dependent on the rate of decay of the bacteria or viruses in the vaccine, as indicated by the stability test.

All veterinary biological products administered to animals should be tested for safety and, if possible, for efficacy in the field, using good clinical practice, before being authorized for general use. Field studies are designed to demonstrated efficacy under working conditions and to detect unexpected reactions, including mortality that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable. The tests should be done on the host animal, at a variety of geographical locations using appropriate numbers of susceptible animals. The test animals should represent all the ages and husbandry practices for which the product is indicated; unvaccinated controls must be included. The product tested should be one or more production batches/serials.

Literature Review

A. Bovine arboviruses overview

The arthropod-borne viruses (arboviruses) are transmitted by blood-sucking arthropods, such as mosquitoes, *Culicoides* biting midges and ticks (Yanase et al., 2005). Arboviruses are classified into 5 families according to their antigenic properties: *Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae,* and *Togaviridae.* Aino virus (AINOV) and Akabane virus (AKAV) in the family *Bunyaviridae* are among the arboviruses that cause disease outbreaks in cattle (Akashi et al. 1997; Uchinuno et al. 1998). Bovine ephemeral fever virus (BEFV) is a member of the family *Rhabdoviridae* (Uren et al., 1992). whereas Chuzan virus (CHUV) and Ibaraki virus (IBAV) belong to the family *Reoviridae* (Jusa et al. 1994; Yanase et al. 2005).

A part of the large complex group of arboviruses in the Bunyaviridae family with over 300 members distributed over five genera. Two of the genera of veterinary importance in the family are Nairovirus and the largest genus, Orthobunyavirus, which is subdivided into 48 serogroups. This genus contains only a few viruses that are significant pathogens of animals such as AKAV. Although they have been placed in different antigenic groups, the viruses have a tropism for fetal tissues and are responsible for embryonic and fetal losses and multiple congenital deformities in domestic ruminants. Members of the Orthobunyavirus genera are single-stranded, enveloped spherical or pleomorphic RNA viruses, 80-110 nm in diameter.

The incidence of arbovirus infection is widely distributed throughout the world. Arboviruses are responsible for many reproductive disorders, including abortion, stillbirth and congenital malformation in ruminants, which produces considerable economic loss in the cattle industry (Akashi et al., 1997a, 1997b, Ohashi et al., 2004; Jago et al., 1993; Olson et al., 2002; Kamata et al., 2009).

Based on information from USDA National Animal Health Reporting System of pilot studies and more recent national studies, the cost of reproductive diseases and conditions was estimated for beef and dairy cattle (Table I). It shows the total yearly cost of female infertility, abortions/stillbirths, dystocia, retained placentas, and metritis/pyometra to be $441 to $502 million for beef producers and $473 to $484 million for dairy producers with an aggregate national total of approximately $ 1 billion annually. This loss is over six times more costly than that resulting from respiratory diseases (Bellow et al., 2002).

Table I. Cost of reproductive diseases and conditions in Cattle (Bellows et al., 2002).

Conditions	Cost (million, $)
Abortion	91
Retained placenta	57
Metritis/ Pyometra	43
Infertility	137
Dystocia	220
Total	548

A.1. Aino virus

Aino virus is member of the genus O*rthobunyavirus*, family *Bunyaviridae*. (Akashi et al., 1997a, 1997b, 1999; Weber et al., 2002). In the former classification (Calisher, 1996), this virus comprised the Simbu serogroup with about 20 other viruses, including Akabane. It is transmitted by hematophagous arthropod vectors, such as *Culicoides* biting midges and mosquitoes, and is widely distributed in Asia and Australia (Brenner et al., 2004; Miura et al., 1982; Yanase et al., 2005). However, the virus' pathogenic importance had not been well reported until a large epidemic of congenital malformation in newborn calves was observed in the southwestern part of Japan from autumn 1995 to spring 1996. After the outbreak, the antigen of Aino virus was immunohistochemically detected and also isolated in the brain of a naturally aborted bovine fetus (Noda et al., 1998; Uchinuno et al., 1998). Yamakawa et al., reported that the Aino virus were grouped into two distinct clusters by analysis based on the N protein sequences. One group consisted of Japanese isolates of Aino, Kaikalur and Shuni viruses and the other group contained Aino virus strain B7974, Australin strain, and Peaton, Sango viruses (Yamakawa et al., 2008). Genetic characterization of Aino and Peaton virus field isolates reveals a genetic reassortment between these viruses in nature (Yanase et al., 2010).

A multiplex real time RT-PCR has also been described using Taq Man probes which is said to reliably identify AINOV and AKAV accurately (Stram et al.,2004).

In Korea, Aino virus infection was first reported in 1997 when neonatal deformities were observed in newborn calves in southern part of Korea (Cho et

al., 2009). Multiplex RT-PCR with Aino viru and Akabane virus and sero-survey on Aino, Akabane, Chuzan, bovine ephemeral fever and Japanese encephalitis virus of cattle and swine were reported (Cho, et al.,2009; Lim et al., 2007). In further study, characterization of the molecular epidemiological analyses of the teratogenic Aino viuse and pathogenic importance should be also conducted in order to understanding of the properties and exact evolutionary relationship.

A.2. Akabane virus

Akabane virus, an arbovirus, causes infection in ruminants including sheep and goats. A member of the genus *Ortho*b*unyavirus,* it falls into the Simbu group. Other *Orthobunyaviruses* that are potential pathogens are Aino (Shuni virus serogroup), Peaton (Shamonda virus serogroup), Douglas (Sathuperi virus serogroup). Akabane virus is however, the best studied and most pathogenic of the Orthobunyaviruses and a major case of arthrogryposis and hydraencephaly. The virus can be isolated from the blood of viraemic animals and occasionally from fetal material. Cell lines of monkey, baby hamster and mosquito are used. The first produces deformities in the developing chicken embryo. Yolk sac inoculation, as well as intracerebral inoculation of suckling mice, is used. Virus or antigen is identified by fluorescence-antibody

AKAV is widely distributed in the tropical and temperate zones in Australia (Hartley et al., 1977; Jagoe et al., 1993), Southeast Asia (Miura et al., 1982), East Asia (Konno et al., 1982), the Middle East (Brenner et al., 2004) and Africa (Metselaar and Robin, 1976; Amira et al., 2014).

In Australia infection among livestock occurs in areas infested with the biting

midge *Culicoides* brevitarsis, an insect from which the virus has been isolated (Cybinski et al., 1984).

In Sudan, Amira et al., reported that AKAV infection is highly prevalent in sheep, goats and dairy cattle by serological survey (Amira et al., 2014).

AKAV was first isolated in Japan in 1959, initially from a mosquito pool and then a pool of *Culicoides* midges. This was followed in 1972 by isolations from *Culicoides* in Australia and mosquito pool isolations in Africa. Akabane virus antibodies have been demonstrated in sera from cattle, sheep, goats, horse, buffalo and camels. Many indigenous game species in Africa south of the Sahara have AKAV neutralizing antibodies. The range of AKAV includes the Middle east, Asia, Cyprus and Africa, but it is in Australia and Japan where regular epizootice of Akabane virus disease occur. Conditions favourable to such outbreaks are susceptible animals in early pregnancy and a sudden increase in vector populations, particularly when the virus has been absent from the area for a number of years.

AKAV infection in adult animals is usually subclinical, but encephalomyelitis has been recently associated with AKAV infection in adult cattle (Lee et al., 2002). Cattle seroconvert after a 3-4day viraemia.

In endemic areas, antibody in the female animal prevents fetal infection, but AKAV is capable of establishing a long-term infection of the placenta in susceptible cattle and sheep. This takes place between 30 and 70 days gestation in the ewe and between 30 and 150 days gestation in the cow. AKAV has a predilection for brain, spinal cord and muscle cells where non-inflammatory necrosis interferes with morphogenesis. AKAV infection has been studied experimentally in sheep and goats with the production of

arthrogryposis/hydroencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (Parsonson et al., 1975). Natural infection of the ovine fetus has been described in Australia where perinatal lamb mortality and congenital microencephaly were most often seen. Experimental AKAV studies have been carried out in pregnant cattle and it was shown that the type of abnormality is dependent on the gestaional age of the fetus with hydroencephaly seen at 76-104 days and arthrogryposis at 103-174 days gestation (Kirkland et al., 1988). This time differential in appearance of abnormalities is clearly seen in bovine fetuses, whereas in sheep with a shorter gestation period, brain and skeletal lesions appear concurrently in the same fetus. The sequence of events during an epizootic of AKAV-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogryposis and dysplastic muscle changes, and lastly those with hydrocephalus and other severe CNS lesion. These events may be preceded by stillbirths and abortions. AKAV is responsible for severe neural and muscular abnormalities and lesions are characterized by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy proencephaly, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necorsis and polymyositis in the myotubules with parenchymal degeneration of skeletal muscles.

Diagnosis of infection is rarely made by virus isolation, but rather by histopathology and serology. Virus has however been isolated from viraemic sentinel animals using plasma or buffy coat suspensions from vector pools and occasionally from fetal material. RT-PCR has been described for the detection of AKAV and differentiation from AINOV. Use of this method could contribute to

diagnosis, but the diversity of the Orthobunyavirus genus will require validation to confirm specificity of the test as there is evidence of reassorment.

Suckling mice, 1-2 days old, may be used and inoculated intracerebrally with 0.01ml of a clarified 10% suspension of the test material. A sensitive system for recovery of this serogroup of viruses is intravenous inoculation of embryonating chick eggs, followed by passage of harvested material in insect cells (C6/36) then mammalian cell lines (Gard et al., 1988; Weir, 2003).

If C6/36 mosquito cells are used, cultures are left stationary for 7 days and material is repassaged on to a hamster or Vero cell line where CPE in the cultures become visible.

In Korea, the first isolation, identification and epidemiological study of Akabane virus were reported from calves with malformations in JeJu (Kang et al., 2000). Susbequently, with Korean isolate Aino and Akabane virus, multiplex RT-PCR was developed (Cho et al., 2009). 14 outbreaks of Akabane disease have been reported from 2002 to 2006 in Korea (Forman et al., 2008). Also, in 2002, Akabnae viral encephalitis of adult cows was reported in South Korea (Lee et al., 2002).

AKAV infection of adult cattle causes a transient viraemia without obvious clinical signs, while infection of pregnant cattle often causes fetal damage resulting in abortion, stillbirth in cattle and congenital abnormalities in calves. The virus replicates in cell culture systems such as baby hamster kidney cell line (BHK-21), vero cells, producing a cytopathic effect.

The disease can be reproduced experimentally in cattle and goat fetuses by placental infection and also by intracerebral inoculation of calves. The encephalitogenic effect of Akabane virus on mice, hamsters, and guinea pigs has

been described by Japanese researchers (Nakajima et al., 1980; Saito et al., 1981).

The genome of orthobunyaviruses comprises three segments of single-stranded, negative-sense RNA, designated on the basis of their distinctive sizes as L (large), M (medium) and S (small) (Nichol et al., 2005). The L RNA segment encodes the L protein, which contains RNA polymerase activity for replication and transcription of the viral genome. The M RNA segment codes for a precursor protein, which is processed into two surface glycoproteins (Gn and Gc) and a nonstructural protein (NSm), is processed by post-translational cleavage. The glycoprotein are responsible for viral neutralization, hemagglutination and attachment to the host cell receptors, which the NSm protein seems to be involved in the process of virus assembly and morphogenesis (Shi et al., 2006). The S RNA segment encodes a nucleocapsid (N) and a non-structural protein (NSs) in an overlapping open reading frame to that encodeing N ptorein. Actually one of the three segments, the M RNA segment encoding Gc, suggesting that its sequence has been subjected to evolutionary pressure by the immunogical systems of vertebrate hosts, is the most variable among orthobunyaviruses (Saeed et al., 2001; Nunes et al., 2005; Briese et al., 2006).

The L RNA segment of the OBE-1 strain was sequenced, while the L RNA segment of AKAV remains uncharacterized (Ogawa et al., 2007). The S and M RNA segments of the AKAV strains have been completely sequenced and reported (Akashi et al., 1997b; Yanase et al., 2003; Kobayashi et al., 2007; An et al., 2010). By phylogenetic analysis of the S and M RNA segments, AKAV were recently divided genetically into four groups (I-IV) and one subgroup (Ia and Ib) (Yamakawa et al., 2006; Kono et al., 2008). Genogroup Ia mainly includes isolates

found in Japan and Taiwan (PT-17, CY77 and NT-14). An Israeli strain (ISR-01) and several Japanese strains are placed in Genogroup 1b (Yamakawa et al., 2006). Genogroup II primarily includes isolates from Japan and Korea (An et al., 2010). Recently, Oem et al., revealed that the AKAV-7 and AKAV-17 strains isolated from a large-scale outbreak of Akabane viral encephalomyelitis belong to genogroup Ia. These isolates most likely had originated from the Iriki strain, which were neurovirulent and caused neurological signs (Oem et al., 2012).

A.3. Bovine ephemeral fever virus

Bovine ephemeral fever (BEF), caused by the bovine ephemeral fever virus, is an acute disease of ruminants (cattle, camel and buffalo) characterized by fever, depression, lamenesss sometimes paralysis called “three day sickness”(Spradbrow, 1986; Uren et al., 1992). The causative virus is a member of the Rhabdoviridae (Della-Porta et al., 1979) and of the genus *Ephemerovirus.* It is heat-labile, ether senstivie and quite susceptible to changes in pH. Virions of the BEFV consist of approximately 70 x 180-nm bullet, enveloped particles. A certain degree of morphologic variability has been recognized in that some particles tent to be bullet-shpaed, whereas others have a more cornical appearance (Della-Porta et al., 1979). The structural proteins also contain a matrix (M) protein and a class I transmembrane glycoprotein (G). The G protein is the target for the neutralizing antibody (Walker, 2005). BEF is not transmitted by close contact, bodily secretions or aerosol droplets (Nandi and Negi, 1999; Yeruham et al., 2010). Most strains were isolated from cattle and the agent has been isolated from various speices of midges and mosquitoes (Venter et al., 2003).

In Japan, the first epidemic of BEF was reported in 1953 and then occurred frequently in the 1960s, but no BEFV activity has been recongnized since 1992 except for Okinawa Prefecture. Niwa et al., isolated BEFV from one of the affected cows' blood samples with clinical sign such as anorexia, fever, decreased activity and compared the complete genome sequence of the G gene. The BEFV isolate, ON-3/E/12, was sorted into the same cluster as other BEFV isolated in Japan, Taiwan and China obtained in 1996-2004 and was most closely related to a 2002 Chinese isolate, JT02L (Niwa et al., 2015).

In Korea, BEFV live vaccines have been commercially available since the 1990s. To date, vaccines for BEF have been actively used, because the vaccines with Akabane diseases are subsidized by the Korean government. Annually, 300,000 doses of BEF vaccine have been provided in areas with a high risk of disease outbreak (Shin et al., 2009).

A.4. Chuzan virus

The family Reoviridae consists of three genera now recognized by the International Committee on Taxonomy of Viruses: *Reovirus*, *Rotavirus* and *Orbivirus*. Chuzan virus belongs to the Palyam serogroup within the genus *Orbivirus* in the family *Reoviridae* (Ohashi et al., 2004; Yamakawa et al., 1999a). They are transmitted by a variety of hematophagous arthropod vectors and have been isolated in the tropical and subtropical regions of the world including Japan, Korea (Knudson et al., 1984; Miura et al., 1988a; Whistler and Swanepoel, 1988). Since 1970, several members of Palyam serogroup have been isolated repeatedly from aborted bovine fetuses (Whistler and Swanepoel, 1988). However, unlike

other orbiviruses, bluetongue virus, epizootic haemorrhagic disease virus and African hosesickness virus, their pathogenic importance had not been well examined until an epizootic of congenital abnormalities with hydraencephaly-cerebellar hypoplasia syndrome of calves was observed in Japan from November 1985 through April 1986 (Goto et al., 1988a, b). Although the Palyam serogroup viruses seem to be contained in producing abortion and congenital malformations in cattle, the pathogenic importance of most of them remains unknown. The genomes of Palyam serogroup viruses consist of 10 dsRNA segments encoding seven structural (VP1-VP7) and four non-structural (NS1, NS2, NS3 and NS3A) proteins (Eaton and Gould, 1987; van Staden and Huismans, 1991). However, very little is known about the genetics and molecular properties, including sequence information, of the Palyam serogroup.

Yamakawa et al., reported that the nucleotide sequence of the entire genome of Chuzan virus has been completed by analysis of the genes encoding minor core proteins (VP1, VP4 and VP6) and non-structural proteins (NS1, NS2 and NS3). The genome of Chuzan virus is 18915 bp in length and the coding capacity of its open reading frames is 6071 amino acid. Comparative sequence analysis with other serogroups of the genus Orbivirus indicated that the outer capsid protein VP2, which is the neutralizaing antigen, reveals to be the most variable and the major core protein VP3 is the most conserved. Overall, the structural proteins, with the exception of VP2 are more conserved than the non-structural proteins among Orbiviruses. Chuzan virus is phylogenetically most related to African horsesickness virus (Yamakawa et al., 1999b).

In Korea, Park et al., first reported an outbreak of Chuzan disease and inoculated

binary ehylenimine-treated Chuzan virus vaccine in cattle (Park et al., 1993). A trivalent inactivated vaccine for Aino virus infection, Akabane diseases, Chuzan diseases was developed in 2011 and has just been released on to the market (Kim et al., 2011).

A.5. Ibaraki virus

Ibaraki disease is cuased by the Ibaraki virus (IBAV) which belongs to the gens *Orbivirus* in the family *Reoviridae* (Suzuki et al., 1977: 1978). Bovine *Orbiviruses* are widespread in nature, and their incidence in cattle is common. Ibaraki virus causes a disease similar to bluetongue in cattle in Japan. Antibodies to Ibaraki virus are common in cattle in all regions of the United States except the northeastern states (Anderson and Campbell, 1979). There is no antigenic relation between bluetongue virus and Ibaraki virus.

The IBAV genome is composed of 10 double-stranded (ds) RNA segments in a bilayered capsid. Segments 3 and 7 encode the inner capsid proteins VP3 and VP7, respectively. These capsid proteins play the role of serogroup-specific antigens. Segments 2 and 6 encode the outer layer proteins VP2 and VP5, respectively, of which VP2 is a major neutralizing antigen and serotype-specific determinant (Huismans et al., 1981).

Its infection is mediated by biting midge and it cause Ibaraki disease appeared by the paralysis of swallowing muscles and abortion in cows. The disease was initially reported in Japan in 1959 (Omori et al., 1969), and since then there have been epidemics of it in east Asia, Australia, Taiwan and Canada (Ohashi et al., 2002: Uchinuno Y et al., 2003). Phylogenetic tree and partial nucleotide sequence

analysis of RNA segment 3 were conducted to compare the IBAV strains from the outbreak regions. Each strain was classified relative to the Ibaraki disease epidemics, which occurred in 1959-1960, 1987 and 1997-1998. IBAV isolated in Japan were more closely related to Taiwanese and Australian strains based on genetics, while the Canadian strain was more distantly related (Uchinuno et al., 2003).

In Korea, Bak et al., first reported that an outbreak of Ibaraki disease (Bak et al., 1983) and eight strains of IBAV were isolated in 1993 (Park, 1993). A vaccine for Ibaraki disease was developed in 2001, but is not actively used, because there has been no known disease outbreak and may pass unnoticed, if there is no special feature (Shin et al., 2009).

B. Control of bovine arboviruses

B.1. Vector and disease control

Culicoides biting midges are the major arthropod vectors for bovine five arboviruses and the most diverse of the 32 genera of Ceratopogonidae. Accurate identification of Culicoides species can be crucial in vector competence studies because the vectorial efficiency for a given virus can vary dramatically between closely related similar species.

Definitive diagnosis both of species and of higher taxa is made mainly on the basis of head spemathecae and male genitalic features. Wing character states, however, are widely used for preliminary sorting of collections and in species diagnoses and keys (Wirth et al., 1985, 1988; Wirth and Hubert, 1989) (Figure I).

Mellor et al. (2000) list more than 50 arbovirus entities isolated from Culicoides species worldwide although only one of these, Oropouche virus, is associated with a clinical syndrome in humans (Pinheiro et al., 1981, 1982, 2004). It is as vectors of virues in livestock however, that *Culicoides* attract special attention. Species in the genus are proven vectors of a number of pathogenic viruses including bluetongue, African horse sickness, epizootic haemorrhagic disease, equine encephalosis and teratogenic viruses, including Akabane and Aino (Mellor et al., 2000).

Accurate figures on the economic impact of Culicoides species on people and livestock enterprises are difficult to establish. It has been estimated that Bluetongue virus causes losses of US $3 billion a year to trade in animals and animal products. This estimate, however, predates the recent dramatic expansion of bluetongue virus activity into eastern and northern Europe (Purse et al., 2005; OIE, Disease information). Additional to the direct costs due to animal losses are the costs of restrictions on local and international trade in live animals and germ plasm and the associated quarantine holding periods. The economic impact of *Culicoides* due to human attack is also largely unknown.

Culicoides species live in subtropical to temperate regions in the world and play an important role in transmitting pathogens to veterinary and medical fields (Mellor et al., 2000). Therefore, it is important to investigate the abundance, population, and distribution of these vectors and associated pathogen infection rates.

Akabane virus was isolated from a biting midge, *Culicoides oxystoma*, collected from a cowshed in Kagoshima, Japan (Kurogi et al., 1987). Chuzan virus was also

isolated from *Culicoides oxystoma* in Japan in 1988 (Miura et al., 1988). Thus, *C.oxystoma* was reported to be the most abundant species and a potential vector for bovine arboviruses in Japan (Yanase et al., 2011). In addition, *C. brevitarsis* in the main vector of arboviruses, such as Akabane virus and bluetongue virus, was reported in Australia (Muller, 1995).

In Korea, *C. punctatus* and *C. arakawae* were the primary species collected at cowsheds in the southern part (Kim et al., 2012; Oem et al). Culicoides species are reported to exist in the entire geographical area of Korea and monitored with five bovine arboviral diseases (Shin et al., 2009). Global warming may have a considerable effect on vector distribution, such as *Culicoides* spp. Therefore, continuing investigation of the distribution and seasonal activity of *Culicoides* biting midges is needed to identify the main vector species.

There is an early warning system for Japanese encephalitis (JE) has been practiced. When JE viruses are detected in arthropod vectors, including Culex species, early warning signs are alarmed for JE. With a proper monitoring system, it would be possible to issue an early warning system for bovine arboviral disease using methods for the detection of viral genes in arthropod vectors, such as Culicoides species.

A reliable supply of pure, safe, potent and effective vaccines is essential for maintenance of animal health and the successful operation animal health programs. Immunisation of animals with high quality vaccines is the primary means of control for many animal diseases. Protection can be obtained against most animal viral diseases, and it is hoped the remaining conditions will be controlled in the near future.

The veterinary practitioner demands a vaccination schedule that is simple, timesaving and commercially attractive. Industry met these requirements by development of multivalent preparations that may contain two or three components. In Japan, a multivalent vaccine together with AKAV, AINOV and CHUV has developed for disease control and is available in the field (Kurogi et al., 1978; Hechinger et al., 2013).

Akabane vaccines have been commercially available in Korea since 1990. However, it is reported that positive rates against other arboviruse diseases have also been increasing (Lim et al., 2007; Yang et al., 2008). For protecting not only Akabane virus but also other arboviral-mediated diseases, the development of multivalent vaccine is required in the field circumstances. As an approach, an inactivated trivalent vaccine against an Aino, Akabane and Chuzan virus was developed and evaluated.

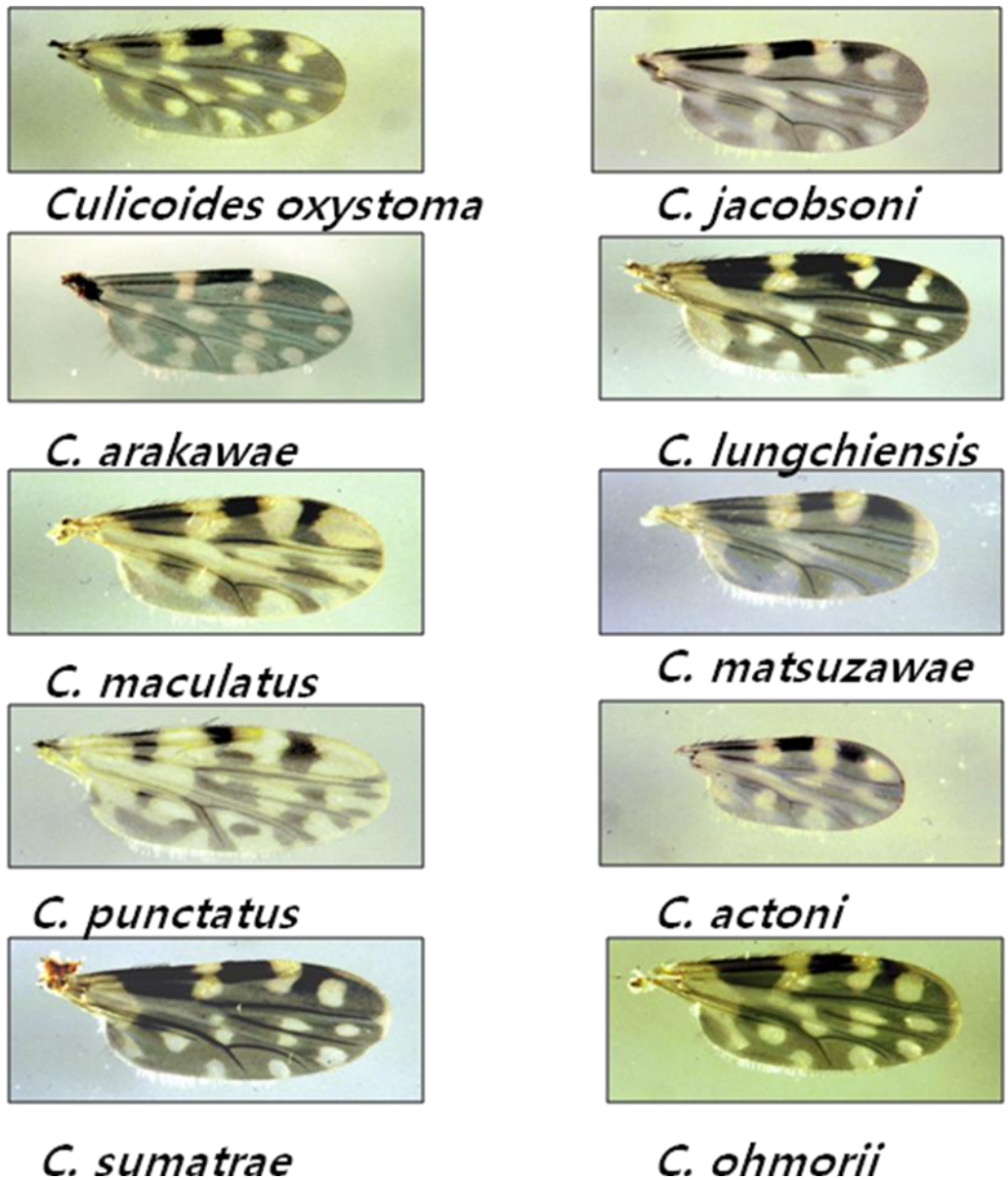


Figure I. Pictorial Atlas of *Culicoides* Wings (Diptera: Ceratopogonidae). (NIAH)

Chapter I

Seroprevalence of five arboviruses in sentinel cattle as part of nationwide surveillance in Republic of Korea, 2009−2012

Abstract

To investigate the possible circulation of arboviruses in Republic of Korea, nationwide surveillance of five arbovirues was conducted in sentinel calves during 2009−2012. We used serum neutralization tests to investigate the presence of antibodies for the Aino virus, Akabane virus, bovine ephemeral fever virus, Chuzan virus and Ibaraki virus. In 2009, 2011 and 2012, the seropositive rates for these five arboviruses were all less than 14.1%. In 2010, however, the seropositive rates for Aino virus and Akabane virus were 33.2% and 40.2%, respectively. High seropositive rates were also associated with a large-scale outbreak of Akabane viral encephalomyelitis in cattle in southern Korea in 2010. Continued seroprevalence surveillance will be useful for monitoring natural arboviral diseases.

Keywords: arboviruses, seroprevalence, South Korea

1.1. Introduction

Arthropod-borne viruses (arboviruses) are transmitted by blood-sucking arthropods, such as mosquitoes, *Culicoides* biting midges and ticks (Yansase et al., 2005). Arboviral infections generally cause not only reproductive disorders, such as abortion, stillbirth and congenital malformation, but also acute febrile disease, resulting in economic losses to the ruminant industry (Lim et al., 2007). Aino virus (AINOV) and Akabane virus (AKAV), in the family *Bunyaviridae*, are among the arboviruses that cause disease outbreaks in cattle (Akashi et al., 1997; Uchinuno et al., 1998). Bovine ephemeral fever virus (BEFV) is classified into the family *Rhabdoviridae* and is known to cause an acute febrile disease called "three day sickness" (Uren et al., 1992). Chuzan virus (CHUV) and Ibaraki virus (IBAV) belong to the family *Reoviridae* and cause reproductive disorders, fever, anorexia and deglutitive disorder (Jusa et al., 1994; Yansase et al., 2005).

Isolation and antibody surveys of arboviruses, including AINOV, AKAV, BEFV, CHUV, and IBAV, have previously been reported in Australia, Asia, Africa and the Middle East (Jagoe et al., 1993; Jun et al., 2012; Lim et al., 2007; Metselaar et al., 1976; Taylor et al., 1994; Yeruham et al., 2010). Outbreaks of the diseases caused by these viruses have been isolated, identified and studied epidemiologically in Republic of Korea (Kang et al., 2000; Park et al., 1993). Since 2007, Akabane viral encephalitis has been reported in calves, and the genetic and pathogenic characteristics of AKAV isolated from cattle with encephalomyelitis have also been described (Lee et al., 2007; Oem et al., 2012). In a 2007 study of thoracic fluids from aborted calves, virus neutralization assays indicated positive rates for AINOV, AKAV and CHUV of 11%, 14.2% and 22.8%, respectively (Lim et al., 2007).

Serosurveillance of AINOV, AKAV and CHUV in Korean native goats (*Capra hircus*) revealed positive rates of 13.3%, 5.5% and 2.0%, respectively (Yang et al., 2008).

Live vaccines for AKAV and BEFV have been commercially available in Republic of Korea since the 1980s. Additionally, an AINOV, AKAV and CHUV trivalent vaccine was developed in 2011 (Kim et al., 2011). However, rates of inoculation with the AKAV and BEFV vaccines are low in Korean native cattle, and the AINOV, AKAV and CHUV trivalent vaccine has received little use in the field. Therefore, it is difficult to distinguish between vaccinated and unvaccinated Korean cattle. In this study, serological monitoring of five arboviruses was conducted during 2009−2012 using a total of 4,000 blood samples, with samples collected from 500 unvaccinated sentinel cattle twice each year. This study represents the first nationwide serological survey of AINOV, AKAV, BEFV, CHUV and IBAV among sentinel cattle conducted by neutralization antibody tests.

1.2. Materials and methods

The study was carried out in all provinces and metropolitan cities of the Republic of Korea. In May and October of each year from 2009 through 2012, blood samples were collected from 500 unvaccinated Korean calves, yielding 1,000 blood samples annually. In May, the sampled cattle were 6−7 months of age. Samples were again collected in October of each year, following the season of vector activity from June to August. These samples were compared to the samples collected from the same individuals in May of that year. The necessary sample size was calculated using Win Episcope 2.0: 475 cattle were required to analyze the

nationwide arboviral prevalence, based on an error of 4.5%, 95% confidence and 50% expected prevalence. Sampling locations are shown in Fig. 1.

The AINOV strain KSA 9910 (Korea Veterinary Culture Collection (KVCC)-VR64), AKAV strain 93FMX (KVCC-VR63), BEFV strain TongRae (KVCC-VR41), CHUV strain YongAm (KVCC-VR66) and IBAV strain 08220 (KVCC-VR65) were used for serum neutralization tests (SNT) (Kim et al., 2011; Lim et al., 2007; Oem et al., 2013). Vero cells (ATCC, C-1586) were maintained in alpha-minimum essential medium (Gibco, Grand Island, NY, U.S.A.) containing 5% fetal bovine serum and antimycotic-antibiotics (Gibco). SNT against AINOV, AKAV, BEFV, CHUV and IBAV were performed in flat-bottomed 96-well plates. Briefly, two-fold serial dilutions of sera were mixed with equal volumes of virus containing 200 $TCID_{50}$/0.1 ml and inoculated with Vero cells (Kim et al., 2011; Lim et al., 2005). The plates were microscopically examined after 3 and 5 days for evidence of virus-specific cytopathic effects (CPE). Antibody titers were expressed as the reciprocal of the highest serum dilution at which CPE was inhibited. A titer of 1:4 or greater was considered to be positive.

1.3. Results

Seropositive animals were those that were antibody-negative in May, but antibody-positive in October of the same year. Therefore, the seroprevalences for the five viruses in this study represent the proportions of the 500 sampled individuals that tested positive for the antibody in each year. Collecting serum samples from the same individuals before and after the vector season provided reliable epidemiological data for arboviral infection.

Among the regions and years studied, Gwangju had the highest seropositivity for AINOV and AKAV at 100% and 66.7%, respectively, in 2010. Gyeongnam had the highest seropositivity for CHUV at 61% in 2010. The highest seropositivity rate for BEFV was 35.7% in Daegu, while that for IBAV was 33.3% on Jeju Island, both in 2011. In 2009, the first year of monitoring, the same individuals could not be sampled a second time in Chungbuk and Jeonnam (Table 1). Therefore, the seropositivity rates for these provinces are not shown in Table 1. No significant association was found between seropositivity and region. Figure 2 shows the seropositivity rates for each virus in each year. In 2009, no virus had a seropositivity rate greater than 13.3%. However, in 2010, the overall seropositivity rates were 33.2% for AINOV, 40.2% for AKAV, 2.9% for BEFV, 29.1% for CHUV and 7.5% for IBAV. In 2011 and 2012, all seropositivity rates were less than 14.1% (Fig. 2).

1.4. Discussion

We found a high seropositivity rate for AKAV in 2010, suggesting that AKAV infection may have caused disease outbreaks in cattle. Indeed, a large-scale outbreak of Akabane viral encephalomyelitis in cattle aged 4−72 months occurred in the southern part of Korea from late summer to late autumn in 2010. Most of the affected cattle were found in Jeonbuk and Jeonnam provinces (OEM et al., 2012). These data were supported by a previous report indicating that arboviral disease has been suspected to be sporadic outbreaks (Shin et al., 2009). Although elevated antibody prevalence also against AINOV in 2010 was founded, AINOV which was associated with the large-scale outbreak including samples of the southern part was

not detected.

Significant seroconversions against AKAV, AINOV and CHUV were observed in 2010. However, no clinical cases were reported in the field for any virus, except for AKAV during the study period. Many other factors may be involved in this result, including characteristics of subclinical signs in most arbovirus-infected cattle. In AINOV and CHUV infections of cattle, *in utero* infections of pregnant cows are common during outbreaks. Therefore, laboratory-confirmed cases are rare in many countries. Additionally, a sporadic outbreak in the field may not be reported. Although unlikely, cross reactivity with other related arboviruses in the virus neutralization test cannot be eliminated as a factor. Therefore, further studies are necessary to reveal the relationship between disease incidence and the seroconversion of sentinel animals.

In this study, we established a statistically reliable sampling strategy for the serological surveillance of arboviruses in Republic of Korea. The sample size used in this study was optimal for the surveillance of herd immunity to arboviruses across the country.

Baseline serological studies of animals, such as the current study, can be used to determine antibody seropositivity rates. The determination of seropositivity rates often leads to an understanding of virus circulation dynamics and is useful in the formulation of disease control measures (Batista et al., 2012). Therefore, quarantines and the summer control of vectors could be better implemented in those provinces with elevated seropositivity rates. Indeed, after the large-scale outbreak in 2010, the Animal and Plant Quarantine Agency (QIA) recommended

preventive vaccination in all regions of Republic of Korea.

In Republic of Korea, Shin *et al.* monitored bovine arboviral diseases using a method for detecting viral genes from arthropod vectors, such as *Culicoides* species, in 2006−2008 (Shin et al., 2009). However, as data from the continued monitoring of arthropod vectors have not been reported since 2009, we are unable to speculate regarding the epidemiological relationship between the viral genes from these arthropod vectors and the seropositivity rates observed in the present study. The species of the biting midge of the genus *Culicoides* (Diptera: Ceratopogonidae) most commonly collected on cattle farms was *Culicoides (C.) punctatus*, followed by *C. arakawae* (Oem et al., 2013). This result is consistent with those of previous seasonal abundance observations of cowsheds conducted in the southern part of the Republic of Korea (Kim et al., 2012). However, the isolation of bovine arboviruses from C*ulicoides* biting midges and the identification of the main vector species remain subjects for a future study. The results of this seroprevalence study may serve as a basis for future epidemiological studies of arboviral infection.

Table 1.1. Percentage of seropositivity from Korean sentinel cattle to each five arboviruses in 2009−2012, presented by region

	Aino virus				**Akabane virus**				**Bovine ephemeral fever virus**				**Chuzan virus**				**Ibaraki virus**			
Region\Year	’09	’10	’11	’12	’09	’10	’11	’12	’09	’10	’11	’12	’09	’10	’11	’12	’09	’10	’11	’12
Busan	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0
Chungbuk	−	15.4	3.4	3.6	−	20.8	11.9	10.9	−	4.0	3.4	0.0	−	34.0	10.2	9.1	−	14.3	13.6	3.6
Chungnam	43.6	20.0	17.8	7.4	0.0	39.3	13.3	13.0	0.0	2.0	6.7	13.0	0.0	21.9	0.0	5.6	1.8	11.6	11.1	3.7
Daegu	0.0	27.3	0.0	27.3	0.0	14.3	0.0	0.0	0.0	0.0	35.7	0.0	0.0	10.0	0.0	9.1	0.0	0.0	0.0	0.0
Daejeon	10.0	75.0	0.0	0.0	0.0	0.0	20.0	10.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gangwon	2.3	40.0	2.9	0.0	2.3	25.0	5.7	5.9	0.0	0.0	2.9	5.9	0.0	15.0	0.0	2.0	0.0	5.4	14.3	0.0
Gwangju	0.0	100.0	0.0	14.3	0.0	66.7	6.7	14.3	0.0	0.0	13.3	0.0	0.0	37.4	0.0	0.0	10.0	0.0	13.3	0.0
Gyeongbuk	0.0	36.0	8.0	6.0	2.0	24.0	2.0	5.0	10.0	12.8	6.0	0.0	0.0	34.1	0.0	2.0	4.0	5.0	18.0	2.0
Gyeonggi	12.7	40.0	10.7	8.0	3.2	44.1	21.4	20.0	0.0	1.7	3.6	0.0	1.6	20.8	7.1	2.0	1.6	7.7	10.7	4.0
Gyeongnam	22.5	51.9	8.0	8.0	11.3	31.7	8.0	8.6	2.8	5.4	8.0	0.0	1.4	61.0	4.0	2.0	1.4	11.5	20.0	2.0
Incheon	0.0	28.6	0.0	0.0	0.0	36.3	0.0	7.7	0.0	0.0	0.0	0.0	0.0	21.4	0.0	0.0	0.0	0.0	0.0	7.7
Jeonbuk	9.1	46.2	2.4	3.3	0.0	34.8	9.5	5.0	0.0	0.0	0.0	6.7	3.0	28.1	2.4	1.7	0.0	3.2	21.4	3.3
Jeonnam	−	42.3	16.3	11.4	−	56.3	2.3	8.6	−	0.0	16.3	17.1	−	38.5	11.6	8.6	−	5.6	7.0	0.0
Jeju	10.0	26.7	3.7	3.3	3.3	14.3	11.1	26.7	0.0	0.0	0.0	6.7	3.3	0.0	7.4	3.3	3.3	7.4	33.3	3.3
Ulsan	0.0	66.7	0.0	10.0	0.0	20.0	0.0	10.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0

- ; Chungbuk and Jeonnam had no the same individual’s data to analyze.

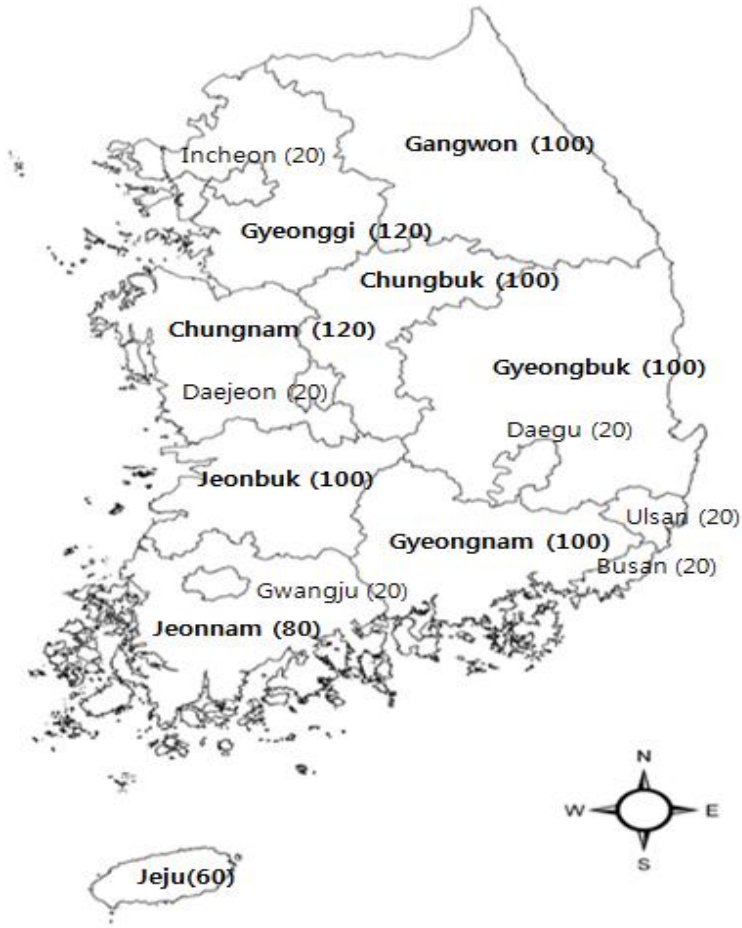


Figure 1.1. Number of cattle sampled in each province of Republic of Korea. Serum was collected from same individual in May and October of each year from 2009 to 2012 for serosurveillance of viruses. Numbers in parentheses represent sample size. Bold characteristics represent the province of Republic of Korea.

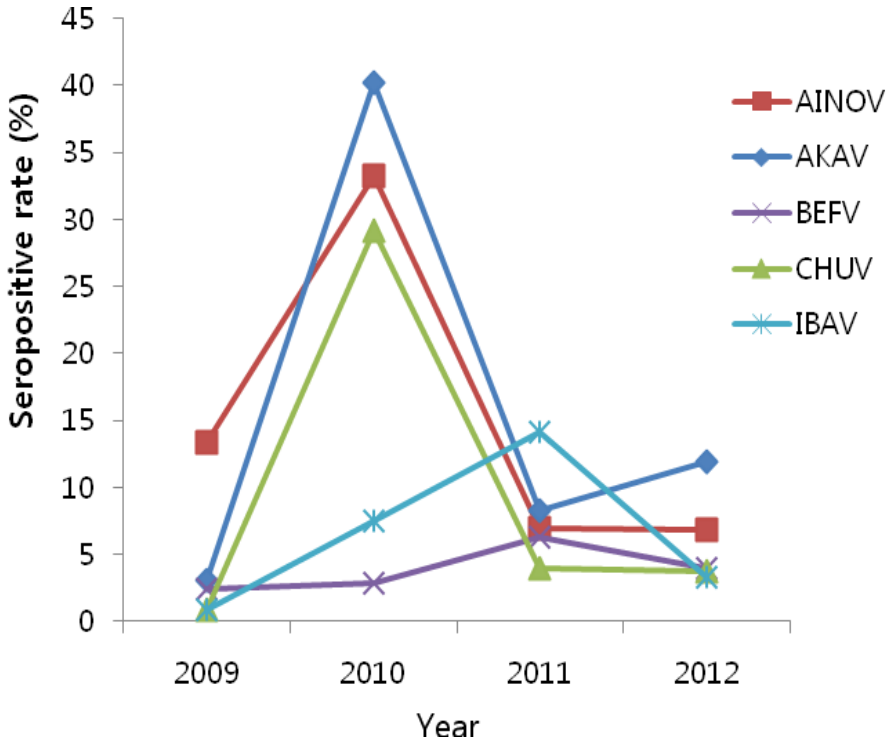


Figure 1.2. Nationwide seropositivity rates for five arboviruses in Republic of Korea from 2009 through 2012. ■, Aino virus; ◆, Akabane virus ; ×, Bovine ephemeral fever virus; ▲, Chuzan virus; ✳, Ibaraki virus.

Chapter II

Development of inactivated trivalent vaccine for the teratogenic Aino, Akabane and Chuzan viruses

Abstract

Aino, Akabane and Chuzan viruses are arthropod-borne (arbo) viruses transmitted by blood-sucking insects like mosquitoes and *Culicoides* biting midges. These arbovirus infections are mainly associated with abortion, stillbirth and congenital defects in pregnant cattle, sheep and goats, which induces a considerable economic loss in livestock industry. The viruses seem to be widely distributed in Southeast Asia and Australia. As a control strategy, an inactivated trivalent vaccine against Aino, Akabane and Chuzan virus was developed by using binary ethylenimine or formalin as an inactivating agent. The newly developed trivalent vaccine is evaluated for its safety and immunogenicity in animals such as mice, guinea pigs and cattle. The immune responses were significantly detected within 2-weeks after second vaccination without any side effects. Since the field application of experimental vaccine also revealed increased antibodies in inoculated cattle, we demonstrated that these trivalent vaccines could be used as a vaccine to control the arboviral infections in ruminants.

Key words: Aino, Akabane, Chuzan virus, Trivalent vaccine

2.1. Introduction

Aino, Akabane and Chuzan viruses are arthropod-borne (arbo) viruses transmitted by blood-sucking insects like mosquitoes and *Culicoides* biting midges (Jennings and Mellor, 1989; Jusa et al., 1994; Uchinuno et al., 1998). Aino and Akabane viruses are members of the genus *Orthobunyavirus*, family *Bunyaviridae* (Akashi et al., 1997a, 1997b, 1999; Weber et al., 2002), and Chuzan virus belongs to the Palyam serogroup within the genus *Orbivirus* in the family *Reoviridae* (Ohashi et al., 2004; Yanakawa et al., 1999). Aino, Akabane and Chuzan viruses are responsible for many reproductive disorders, including abortion, stillbirth and congenital malformation in ruminants, which produces considerable economic loss in the cattle industry (Akashi et al., 1997a, 1997b; Ohashi et al., 2004; Jagoe et al., 1993; Olson et al., 2002; Kamata et al., 2009).

The first isolation, identification and epidemiological study of Akabane virus were reported from calves with malformations in Jeju (Kang et al., 2000). Subsequently, with Korean isolate Aino and Akabane virus, multiplex RT-PCR was developed (Cho et al., 2009). In addition, Chuzan disease was also confirmed in Korea (Park et al., 1993). 14 outbreaks of Akabane diseases have been reported from 2002–2006 in Korea (Forman et al., 2008). Also in 2007, Akabane viral encephalitis of calves was reported in Republic of Korea (Lee et al., 2007).

A study conducted in Republic of Korea, which examined aborted calves using a virus neutralization assay, reported positive rates for Aino, Akabane and Chuzan viruses of 11%, 14.2% and 22.8%, respectively (Lim et al., 2007). Serosurveillance of viral diseases in Korean native goats (*Capra hircus*) for Aino,

Akabane and Chuzan viruses were also 13.3%, 5.5% and 2.0%, respectively (Yang et al., 2008).

In fact, a total of 1,000 blood samples were routinely collected from sentinel cattle for serological monitoring against 5 arthropod-borne viruses, Aino, Akabane, Chuzan, Bovine ephemeral fever and Ibaraki virus in Korea. Based on the sero-survey results, the early warning recommendations, if necessary, are implemented for preventive vaccination and for controlling arthropod vectors in the field every year.

Any increase in temperature results in an increase in the range, abundance and seasonal activity of *Culicoides* biting midges, which are capable of transmitting the bovine arboviruses (Yanase et al., 2005; Purse et al., 2005). Given the current economic importance of these viral infections, and the prospect that the geographic range of the biting midge will increase by global warming, various protective measures including vaccination have been recognized (Forman et al., 2008; Tsutsui et al., 2009).

Akabane vaccines have been commercially available in Korea since 1990. However, it is reported that positive rates against other arbovirus diseases have also been increasing (Lim et al., 2007; Yang et al., 2008). For protecting not only Akabane virus but also other arboviral-medicated diseases, the development of multivalent vaccine is required in the field circumstances. As an approach, the present study reports the development and *in vivo* evaluation of an inactivated trivalent vaccine against an Aino, Akabane and Chuzan virus.

2.2. Materials and methods

2.2.1. *Virus strains and cell culture*

Aino virus KSA9910 vaccine strain was isolated in Chonnam province in 1999. Akabane virus K-9, 93FMX, S-1 strains were investigated as vaccine candidates. Chuzan virus Youngam strain was isolated in Chonnam province in 1997. All of these strains were isolated from the blood samples collected by adult cows. Each strain was registered in the Biological Resource center (BRC), Korea Research Institute of Bioscience and Biotechnology (KRIBB) as KCTC18118P for the KSA9910 strain of Aino virus, KCTC 18117P for the K-9 strain of Akabane and KCTC18119 for the Youngam strain of Chuzan virus, respectively.

Vero cells (ATCC, C-1586), hamster lung (HmLu-1) and baby hamster kidney (BHK-21) cells were cultured to compare the viral titer of Aino, Akabane and Chuzan viruses after inoculation. The isolates were passaged in 80-90% monolayers of each cells in alpha-minimum essential medium (GibcoBRL, USA) and antimycotic and antibiotics (GibcoBRL, USA). The plates were incubated at 37℃ for 7 days and then each culture fluid was harvested for the infectivity when the maximum cytopathic effects (CPE) were observed. The virus titer was expressed as 50% tissue culture infective doses ($TCID_{50}$) per 0.1 ml, calculated by the Reed-Munch method (Reed and Munch, 1938).

2.2.2. *Inactivation of virus*

0.2% Formalin or 0.001 M binary ethylenimine (BEI) was added to each virus suspension to inactivate the virus (Kolar et al., 1973; Ramakrishnan et al., 2006). Each suspension was shaken sufficiently and kept at 4℃. In order to determine the

time required for complete inactivation, samples were collected from BEI and formalin treated virus suspension at given intervals of time.

2.2.3. *Preparation of vaccine*

For the preparation of vaccine, a strain of Korean isolate of Aino (KSA9910), Akabane (K-9) and Chuzan virus (Youngam) was cloned by using the plaque-purified method once in Vero cells (Lim et al., 2007). Each of Akabane, Chuzan virus was inoculated in the bottle of Vero cells (ATCC, C-1586) and Aino virus was inoculated in the HmLu-1 cell at 1-10 multiplicity of infection (MOI) and maintained in alpha-MEM with 2.5% FBS at 37℃. When CPE in virus-inoculated cells appeared completely, the culture fluid of infected cells was collected and centrifuged at 3,000 rpm for 10 min. In order to prepare trivalent vaccine, equal volume of the inactivated virus solution was mixed. An adjuvant, Montanide® IMS 1314 (Seppic, France) was added to the mixture of three inactivated viruses to a concentration of 50% (v/v). All the vaccines prepared were stored at 4℃ until use (Kurogi et al., 1978).

2.2.4. *Safety test*

For evaluation of safety, specific pathogen-free (SPF) female ICR mice, Hartley female guinea pigs and Holstein-Friesian breed cows at 3-7 months of pregnancy were inoculated with the inactivated trivalent vaccine as described in Table 1. These tests were carried out according to the Korea governmental regulation standard assay for Veterinary Biologicals.

2.2.5. *Serum neutralization test*

Like safety test, guinea pigs and pregnant cows were also used to examine immune response against BEI- or formalin-inactivated vaccine. Hartley female guinea pigs were immunized twice with 3 ml of experimental vaccine intramuscularly at 3-week intervals and blood samples were tested for antibody titers. Twelve guinea pigs were inoculated as the vaccinated group and five guinea pigs were monitored as the controls. A dose of 3 ml was adopted for vaccine inoculation in Holstein-Friesian breed cows. Two groups of five pregnant cows were inoculated with 3 ml experimental vaccine inactivated by BEI or formalin intramuscularly at 3-week intervals. To serve as a control group, phosphate buffered saline, pH 7.4 (PBS) was inoculated at the same intervals. Blood samples were collected from all animals at regular intervals after inoculation. Serum samples were stored at -20℃ until use. Two-fold serial dilutions of sera were mixed with an equal volume of virus containing 200 $TCID_{50}$/ml and were inoculated in 96-well micro plate cultures of Vero cells (Lim et al., 2005). The antibody titer was expressed as the reciprocal of the highest serum dilution inhibiting CPE. Titers of 4 or higher were taken as positive in this study. The results were analyzed by the ANOVA test. A p-value < 0.05 was considered to be statistically significant.

2.2.6. *Field application*

Field application was conducted by using formalin-inactivated vaccine in collaboration with Dae-sung microbiological research laboratory (Republic of Korea). A total of sixty seronegative Holstein-Friesian breed cattle were selected

for field application in five farms without case histories of arthropod-borne viral diseases. Ten pregnant cows were intramuscularly administered with 3 ml vaccine twice at three week intervals. Two cattle were monitored as the control in each farm.

2.2.7. *Challenge exposure*

A total of nine pregnant cows, Holstein-Friesian breed, were inoculated for the challenge exposure at 120 to 180 days of gestation. Six cows were vaccinated with formalin-inactivated trivalent vaccine twice intramuscularly at three-week intervals and three cows remained as the controls. Two vaccinated cows were challenged intravenously with 5 ml of each vaccine strain ($10^{7.0}$ $TCID_{50}$/ml) at 3 weeks after the 2nd vaccination. At the same time, each one out of three control cattle was also challenged in the same route along with vaccinated cows. After challenge exposures, clinical signs were observed for 5 day. Viremia was confirmed by not only RT-PCR but also virus isolation as described previously (Kang et al., 2000, Cho et al., 2009, Ohashi et al., 2004).

2.3. Results

2.3.1. *Propagation and inactivation of viruses*

Aino virus KSA9910 strain achieved the highest titer ($10^{7.5}$ $TCID_{50}$/ml) at eighth passage in HmLu-1 cells, while Akabane virus K-9 strain and Chuzan virus Youngam strain were most efficiently propagated in Vero cells. The highest titers of Akabane virus ($10^{7.3}$ $TCID_{50}$/ml) and Chuzan virus ($10^{6.8}$ $TCID_{50}$/ml) were obtained at ninth and eighth passages, respectively. Theses cell culture supernatants were

used for preparation of the experimental vaccine.

The viral suspension treated with each inactivation reagent was harvested at every hour and tested for infectivity. The infectivity was completely inactivated by 3 h with the final concentrations of 0.2% formalin at 4℃. In the case of BEI, the viral suspension was treated with 2 M sodium thiosulfate to a final concentration of 2 mM for the neutralization of BEI at 37℃. The complete inactivation was detected at 4 h after treatment with BEI.

2.3.2. *Safety test*

Safety experiments of the vaccines were performed on mice, guinea pigs and cows (Table 1). Mice and guinea pigs did not display any clinical sign after inoculation. Eight vaccinated cows with formalin-inactivated vaccine and eight pregnant cows with BEI-treated vaccine also remained clinically normal throughout the experimental period. Pyrexia and any other general symptom were not found in any cow during 56-days post-inoculation.

2.3.3. *Antibody responses*

The serum neutralization (SN) titers against Aino, Akabane and Chuzan viruses were examined after immunization with the BEI- or formalin-inactivated vaccine. Geometric mean of the titers (log_2) of guinea pigs was plotted in Figure 1. SN titers (log_2) of pregnant cows were also plotted in Figure 2. There was significant difference in antibody titers between pre-inoculation, 3-weeks and 5-weeks by ANOVA ($P<0.05$). The stability of the vaccines were also investigated. Both BEI- and formalin-inactivated vaccines were stable for one year at 4^oC with SN titer

being at least more than 8 in guinea pigs as shown in Table 2.

2.3.4. *Field application*

Since the formalin inactivation of viruses turned out to be simple and easy to prepare the vaccine compared to BEI treatment without any significant immunological differences in experiment animals, the formalin treated trivalent vaccine was further selected to apply for the field trial and challenge test in this study. All the inoculated cattle did not show any clinical disorders and delivered the normal calves. After second vaccination, the result showed the overall increased immune responses than first inoculation as shown in Figure 3. SN titer (log_2) was more than 2.8 for Aino, 5.6 for Akabane and 3.7 for Chuzan.

2.3.5. *Challenge exposures*

Although all vaccinated cows did not develop fever, leukopenia or any other clinical abnormalities, the viremia was only possible to detect with the control challenged with Akabane virus, but not with the cows from Aino virus and Chuzan viruses. However, the isolation of Akabane virus in Vero cells was not successful in this study.

Nevertheless, three control cows showed the SN antibody titers against Aino, Akabane and Chuzan viruses, indicating that they had actually acquired virus infection after challenge exposures (Table 3).

2.4. Discussion

In this study, an inactivated trivalent vaccine was investigated for its safety and immunogenicity in mice, guinea pigs and cows. In addition, the sero-conversion of SN antibodies was also assessed from pregnant cows in the field application.

As important factors affecting the antibody response of an animal to vaccine, conditions for inactivation of virus, type of adjuvant, conditions for storage of vaccine, dose and interval of inoculation should be considered (Kurogi et al., 1978).

Recently, Montanide® IMS is a water-based formulation of nanoparticles that can enhance short-term and long-term immunity. No specific process is required to manufacture the vaccines, consisting only of a simple mixing of antigenic medium and nanoparticle solution (Aucouturier et al., 2001). This approach was presently used to generate efficacious and safe vaccines by mixing nanoparticles and inactivated bovine arboviruses.

Mice, guinea pigs and cows are known to be suitable animals for potency testing of vaccines (Kurogi et al., 1978; Goto et al., 1988). Previously, it was reported that an infected cell culture fluid of high titer (exceeding $10^{6.5}$ $TCID_{50}$/ml) is necessary to developing Akabane-inactivated vaccine (Kurogi et al., 1978). At the time of challenge inoculation the SN antibody titer was 2–8 in the vaccinated group and <1 in the control group in a pregnant goat (Kurogi et al., 1978).

Our results indicated that the inactivated trivalent vaccine can simultaneously induce SN antibodies against Aino, Akabane and Chuzan viruses, and can prevent the diseases. Since a twofold titer by SN test indicated the presence of maternal antibodies, seronegative cattle below twofold were also selected for the field application in present study. The results showed that the formalin-inactivated

trivalent vaccine for Aino, Akabane and Chuzan diseases turned out to be safe because there were no clinical disorders after vaccination. Therefore, it is possible to expect that the vaccination induced the overall herd immunity in the farms. When we challenged the pregnant cow, it was demonstrated that the vaccinated and control cows resulted in the presence of increased antibody titer. However, we could not detect any sign of fever and leukopenia from controls challenged with Aino, Akabane and Chuzan virus in this study. And, we could not detect the viremia from Aino and Chuzan virus except Akabane virus from control cows.

One of the possible explanations for these results may presumably attribute to attenuation of viral strains during the passages in cells. Previously, it was reported that pathological properties of Akabane virus were only detected from the strain after *in vivo* passage using suckling mice (Kurogi etla., 1977). For this reason, it is worthy to note that it is preferable to maintain the same strain through *in vivo* passage for the challenge experiment. In addition, if we consider the complicated pathological properties of arboviruses in pregnant animals, the route of challenge exposures could be one of the most critical factors that link direct infection to embryo *in vivo*.

Although further clinical evaluation for the prophylactic efficacy as well as the duration of immunity with statistical determination is necessary in the field, the present results indicate that the formalin-inactivated trivalent vaccine is a promising candidate for a vaccine against arboviral diseases.

Table 2.1. Safety of trivalent vaccines in experimental animals

Trivalent Vaccine	Animal	No.of animals	Dose (ml)	Inoculation route	Period (days)	Clinical sign	
						Pyrexia	General
BEI inactivated	Mice	10	0.5	SC [a)]	14	- [d)]	-
	Guinea pigs	4	5	IP [b)]	14	-	-
	Cows	8	6	IM [c)]	56	-	-
Formalin inactivated	Mice	10	0.5	SC	14	-	-
	Guinea pigs	4	5	IP	14	-	-
	Cows	8	6	IM	56	-	-

a) SC: Subcutaneous.

b) IP: Intraperitoneal.

c) IM: Intramuscular.

d) - : None.

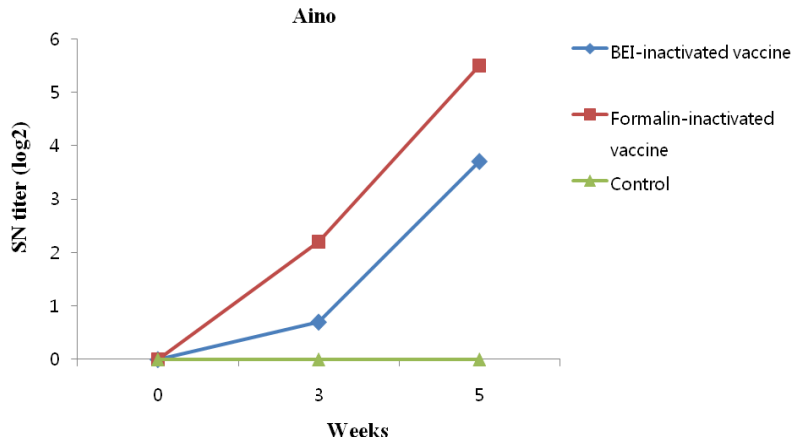
Table 2.2. Stability of trivalent vaccines in guinea pigs

Trivalent vaccine	No.of animals	Virus	SN titer (log_2)	
			6 months	1 year
BEI inactivated	10	Aino	3.5	4.8
		Akabane	5.4	4.8
		Chuzan	3.9	3
Formalin inactivated	10	Aino	3.5	4.6
		Akabane	3.8	4.7
		Chuzan	5	4.8

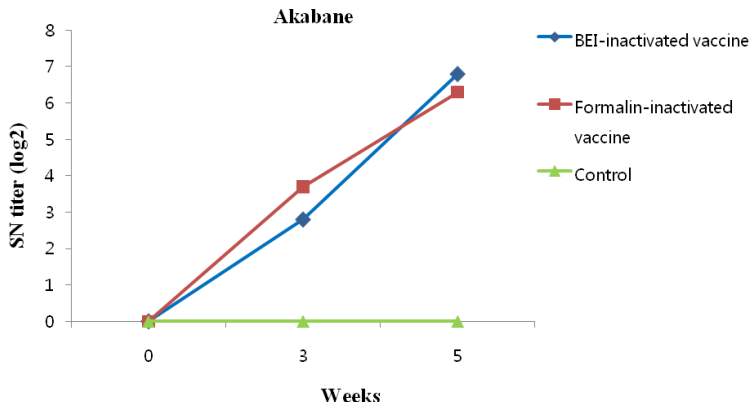
Table 2.3. Antibody responses and viremia of vaccinated and control cows after challenge with Aino, Akabane and Chuzan virus

Group	Cow No.	Before	After 2nd vaccination			Challenged by	After Challenge exposure				
							5 day			Viremia	Virus isolation
		Aino, Akabane, Chuzan	Aino	Akabane	Chuzan		Aino	Akabane	Chuzan	RT-PCR	
Vaccinated	1	<2	16	32	4	Aino	256	128	16	-	-
	2	<2	2	64	2	Aino	128	128	8	-	-
	3	<2	16	16	2	Akabane	256	256	8	-	-
	4	<2	16	16	8	Akabane	256	256	64	-	-
	5	<2	2	2	8	Chuzan	256	256	128	-	-
	6	<2	16	16	8	Chuzan	256	256	64	-	-
Unvaccinated Control	1	<2	<2	<2	<2	Aino	16	<2	<2	-	-
	2	<2	2	<2	<2	Akabane	2	16	<2	+	+
	3	<2	<2	<2	<2	Chuzan	<2	<2	8	-	-

A)


Aino
SN titer (log2)
6
5
4
3
2
1
0
0
3
5
Weeks
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

B)


Akabane
SN titer (log2)
8
7
6
5
4
3
2
1
0
0
3
5
Weeks
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

C)

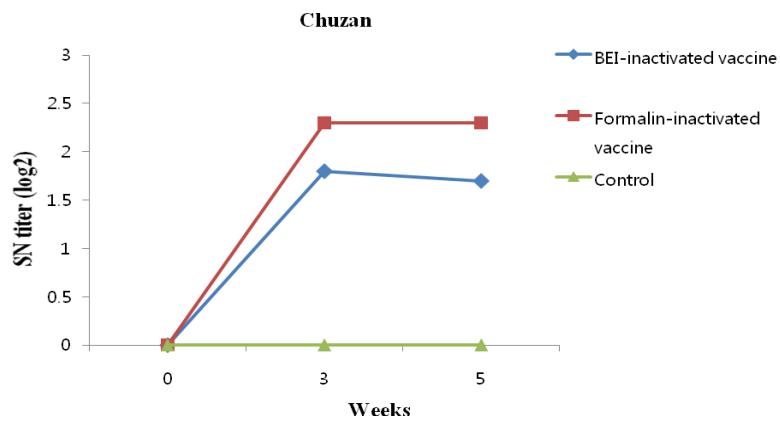
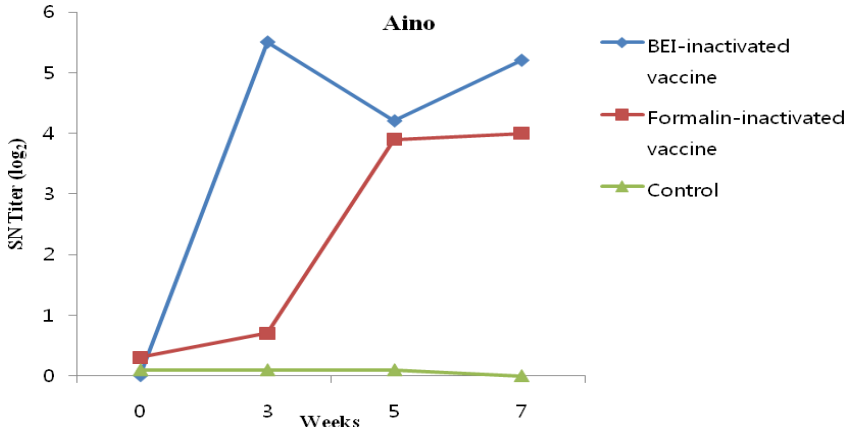

Chuzan
SN titer (log2)
3
2.5
2
1.5
1
0.5
0
0
3
5
Weeks
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

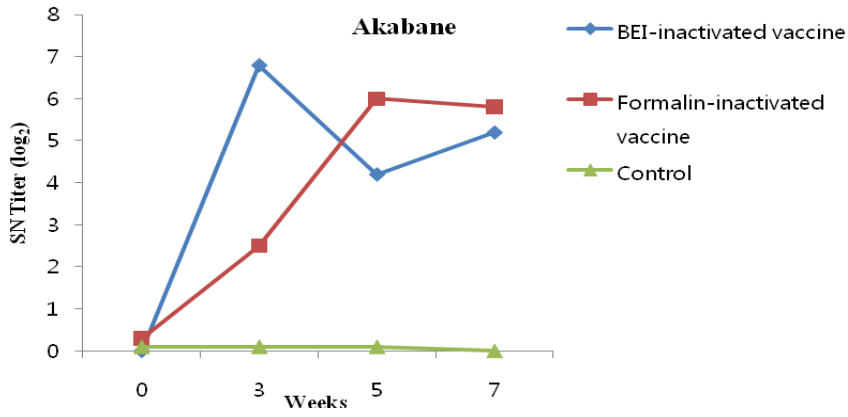
Figure 2.1. Serum neutralization (SN) antibodies from Hartley guinea pigs inoculated with either BEI or formalin-inactivated vaccine. ♦: indicate that the vaccine is inactivated by BEI, ■: indicate that the experimental vaccine is inactivated by formalin and ▲: indicate that the control cows, respectively.

At 0 week is the first inoculation, at 3 weeks is the second inoculation. A) SN titer (log_2) of Aino virus. B) SN titer (log_2) of Akabane virus. C) SN titer (log_2) of Chuzan virus.

A)


Aino
SN Titer (log2)
Weeks
0
3
5
7
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

B)


Akabane
SN Titer (log2)
Weeks
0
3
5
7
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

C)

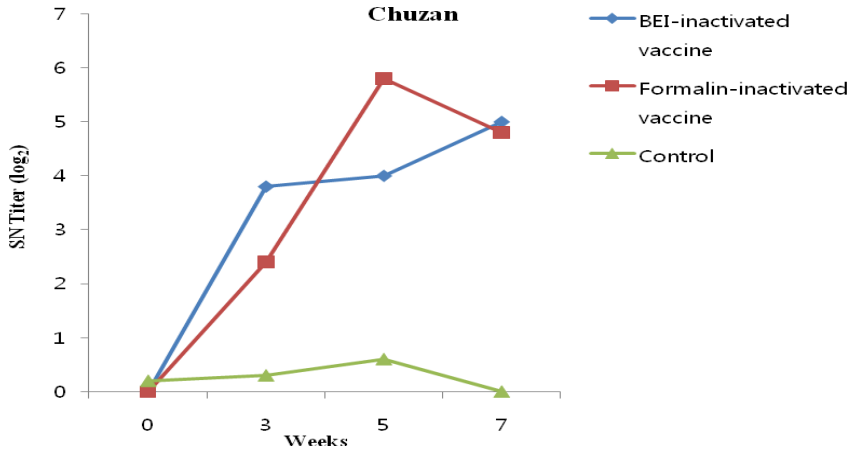
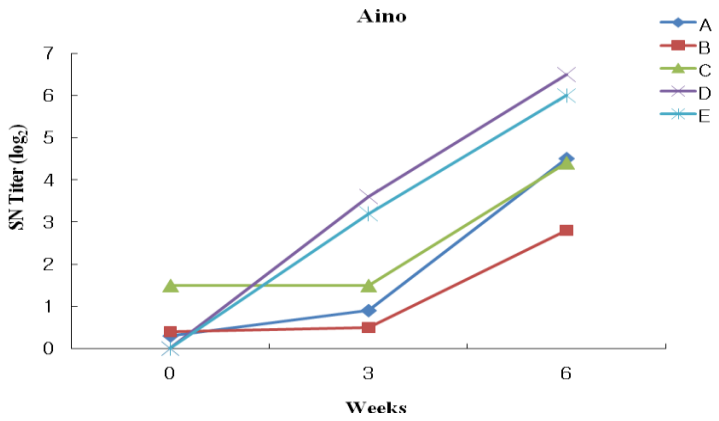
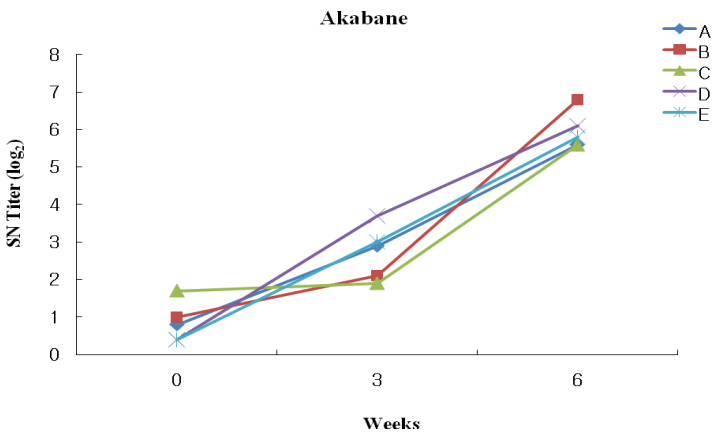

Chuzan
SN Titer (log2)
Weeks
0
3
5
7
BEI-inactivated vaccine
Formalin-inactivated vaccine
Control

Figure 2.2. Serum neutralization (SN) antibodies from pregnant cows inoculated with either BEI or formalin-inactivated vaccine. Three groups of five cows were inoculated in the same farm. ◆: indicate that the vaccine is inactivated by BEI, ■: indicate that the experimental vaccine is inactivated by formalin and ▲: indicate that the control cows, respectively. At 0 week is the first inoculation, at 3 weeks is the second inoculation. A) SN titer (log_2) of Aino virus. B) SN titer (log_2) of Akabane virus. C) SN titer (log_2) of Chuzan virus.

A)


Aino
A
B
C
D
E
SN Titer (log2)
0
1
2
3
4
5
6
7
0
3
6
Weeks

B)


Akabane
A
B
C
D
E
SN Titer (log2)
0
1
2
3
4
5
6
7
8
0
3
6
Weeks

C)

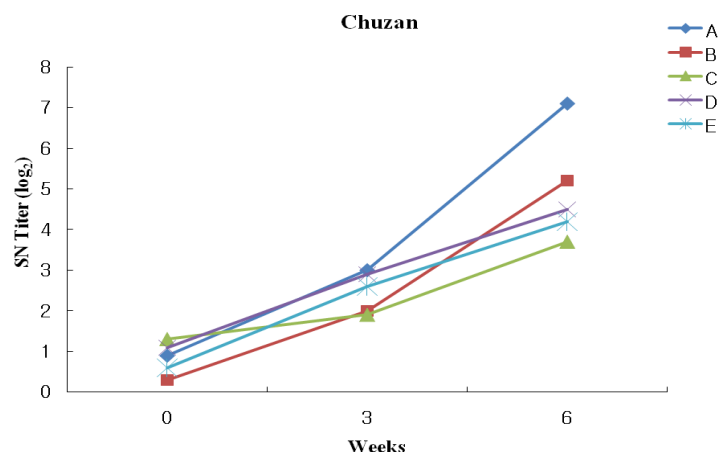

Chuzan
A
B
C
D
E
SN Titer (log2)
0
1
2
3
4
5
6
7
8
0
3
6
Weeks

Figure 2.3. Immune responses of pregnant cows with trivalent vaccine in the field application. Cows were inoculated with formalin-inactivated vaccine in five farms from A to E. At 0 week is the first inoculation, at 3 weeks is the second inoculation. A) SN titer (log_2) of Aino virus. B) SN titer (log_2) of Akabane virus. C) SN titer (log_2) of Chuzan virus.

GENERAL CONCLUSION

The arbovirus infections have been considered to important disease as a warning for global climate warming. In this study, seroprevalence of five arboviruses in sentinel cattle was carried out for the investigation the presence of antibodies for the Aino virus, Akabane virus, bovine ephemeral fever virus, Chuzan virus and Ibaraki virus in South Korea. Serological monitoring of five arboviruses was conducted during 2009-2012 using a total of 4,000 blood samples, with samples collected from 500 unvaccinated sentinel cattle twice each year. In addition, inactivated trivalent vaccine for the Aino, Akabane, and Chuzan viruses was developed.

Among the regions and years studied, a high seropositivity rate for AKAV in 2010 was associated with a large-scale outbreak of Akabane viral encephalomyelitis in cattle in southern Korea. Summer control of vectors, such as *Culicoides* species, and vaccination are considered to be the most efficacious and economic control measure.

Presently, live vaccines for AKAV and BEFV have been commercially available in South Korea since the 1980s. Although the government provides the vaccine to farmer, rates of inoculation with the AKAB and BEFV vaccines are low in Korean native cattle. For protectin not only AKAV but also other arboviral-mediated diseases, the development of multivalent vaccine is required in the field circumstances.

Based on these results, the determination of seropositivity rates often leads to an understanding of virus circulation dynamics and is useful in the formulation of

disease control measures. In addition, multivalent vaccine for protectin arboviral mediated diseases is expected as useful tool in the field circumstances.

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국문초록

5종의 소 아보바이러스 유병률 및 아이노, 아까바네, 츄잔바이러스 3종 불활화 혼합백신 개발

2009-31053 김연희
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아보바이러스는 모기, 등에모기, 진드기와 같이 흡혈하는 절지동물에 의해 전염되는 바이러스의 총칭이다. 소에서 이러한 아보바이러스 감염은 주로 유산, 사산 및 선천적 기형을 유발하며 가축산업에 상당한 경제적 손실을 유발한다. 따라서, 본 연구에서는 2009년에서 2012년까지 국내에 발병보고된 5종의 아보바이러스 (아아노, 아까바네, 소 유행렬, 츄잔, 이바라기 바이러스)에 대해 감시동물을 대상으로 혈청중화시험을 이용하여 유병률을 조사하였다. 등에모기가 나타나는 여름을 전후로 항체 음성에서 양성으로 변하는 항체 양전률을 조사한 결과 2009년, 2011년, 2012년에는 5종 모두 14.1% 미만이였다. 그러나, 2010년에는 아이노바이러스, 아까바네바이러스가 각각 33.2%, 40.2%로 나타났다. 2010년 이러한 높은 항체 형성률은 남쪽지방에서 아까바네바이러스에 의한 뇌염소견으로 대

규모 발병한 사례와도 연관이 있었다.

아보바이러스는 동남아시아 및 호주일대에 널리 분포하고 있는 것으로 알려져 있으며, 국내에서는 아이노, 아까바네, 츄잔바이러스가 각각 유사산 시료에서 분리되었다. 질병예방 대책으로 3종의 혼합백신이 포르말린 불활화 및 BEI 불활화 방식으로 개발되었다. 개발된 백신은 마우스, 기니피그, 목적동물인 소를 이용하여 안전성 및 면역원성이 평가되었다. 면역원성은 2차 접종 2주후에 유의성있게 면역반응을 형성하는 것을 알 수 있었으며 접종 부작용은 없었다. 야외 소 농장 적용 시험을 통해서도 3종 불활화 혼합백신이 질병을 예방하는데 도움이 될 것으로 판단되었다.

이번 연구 결과들을 통하여 아보바이러스의 지속적 혈청학적 유병률 조사는 아보바이러스 감염의 모니터링을 위해 유용할 것이며, 포르말린 불활화 혼합백신은 아보바이러스질병예방에 기여할 것으로 사료된다.

Key words: 아보바이러스, 혈청학적 유병률, 아이노바이러스, 아까바네바이러스, 츄잔바이러스, 3종 불활화 혼합백신

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List of published articles

2015

1. **Yeon-Hee Kim**, Jae-Ku Oem, Eun-Yong Lee, Koung-Ki Lee, Seong-Hee Kim, Myoung-Heon Lee, Se Chang Park (2015). Seroprevalence of five arboviruses in sentinel cattle as part of nationwide surveillance in South Korea, 2009-2012. J. Vet. Med. Sci. 77(2):247-250.
2. **Yeon-Hee Kim**, Eun-Yong Lee, Jae-Ku Oem, Seong-Hee Kim, Myoung-Heon Lee, Koung-Ki Lee, Se Chang Park (2015). Genetic analysis of *env* and *gag* gene fragments of bovine leukemia virus identified in cattle from Korea. Korean J Vet Res. 55(1):53-56.
3. Jae-Ku Oem, Seong-Hee Kim, **Yeon-Hee Kim,** Myoung-Heon Lee, Koung-Ki Lee (2015). Molecular characteristics of canine parainfluenza viruses type 5 (CPIV-5) isolated in Korea. Can J Vet Res 79(1):64-67.
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