Pathogenicity and teratogenicity of Schmallenberg virus and Akabane virus in experimentally infected chicken embryos

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ABSTRACT

Schmallenberg virus (SBV) and Akabane virus (AKAV) are teratogenic Simbu serogroup Orthobunyaviruses. Embryonated chicken egg models (ECE) have been used to study the pathogenicity and teratogenicity of Simbu viruses previously, however to date no such studies have been reported for SBV. Hence, the aims of this study were to investigate if ECE are susceptible to experimental SBV infection, and to evaluate the pathogenicity and teratogenicity of SBV and AKAV in ECE models.

Two studies were conducted. In Study A, SBV ($10^{6.4}$ $TCID_{50}$) was inoculated into the yolk-sac of 6-day-old and 8-day-old ECEs. In Study B, SBV and AKAV were inoculated into 7-day-old ECEs at a range of doses ($10^{2.0}$–$10^{6.0}$ $TCID_{50}$). ECE were incubated at 37 °C until day 19, when they were submitted for pathological and virological examination.

SBV infection in ECE at 6, 7 and 8 days of incubation resulted in stunted growth and musculoskeletal malformations (arthrogryposis, skeletal muscle atrophy, contracted toes, distorted and twisted legs). Mortality was greater in embryos inoculated with SBV (31%) compared to AKAV (19%), ($P < 0.01$), suggesting that SBV was more embryo-lethal. However, embryos infected with AKAV had a significantly higher prevalence of stunted growth ($P < 0.05$) and musculoskeletal malformations ($P < 0.01$), suggesting that AKAV was more teratogenic in this model.

These studies demonstrate for the first time that the ECE model is a suitable *in vivo* small animal model to study SBV. Furthermore, these results are consistent with the clinico-pathological findings of natural SBV and AKAV infection in ruminants.

1. Introduction

Schmallenberg virus (SBV) is a Simbu serogroup virus in the genus Orthobunyavirus (family Peribunyaviridae, order Bunyavirales) that emerged for the first time in north-western Europe during 2011 (Hoffmann et al., 2012). The virus was identified by metagenomic analysis of blood samples collected from dairy cattle in Germany presenting with non-specific clinical signs of fever and drop in milk yield (Hoffmann et al., 2012). Subsequently, SBV was implicated in a pan-European epizootic of abortions, congenital malformations and stillbirths in domestic ruminants (cattle, sheep and goats) in 2012 and 2013 (EFSA, 2013, 2014).

Akabane virus (AKAV) is a phylogenetically closely related Simbu serogroup Orthobunyavirus which was first isolated in Japan in 1959 (Oya et al., 1961), and has since been reported in a number of countries in Africa, the Middle East and Australasia. Schmallenberg virus and

Akabane virus have similar epidemiological characteristics as well as pathogenesis and clinical signs; both are transmitted by *Culicoides* species biting midges (Kurogi et al., 1975; Regge et al., 2012) and infection of ruminants with these viruses during pregnancy (between approximately day 80–150 in cattle and day 28–56 in sheep) can cause foetal infection resulting in abortions and congenital malformations most notably characterised by the arthrogryposis-hydranencephaly syndrome (AHS) (Herder et al., 2012; Oya et al., 1961).

In vivo experimental infection studies have helped elucidate the pathogenicity and teratogenicity of AKAV; experimental infection with AKAV produced congenital malformations, primarily arthrogryposis and hydranencephaly in calves (Kurogi et al., 1977), lambs (Hashiguchi et al., 1978), and goat kids (Kurogi et al., 1976). Experimental infection studies have also been used to investigate the gestational ages at which AKAV may be pathogenic in cattle (Kurogi et al., 1977) and in sheep (Parsonson et al., 1988, 1981b).

In vivo research studies on teratogenic viruses in cattle, sheep and goats are often expensive (for large animals and high containment facilities) and typically require a greater length of time for experiments to be completed due to the longer gestation length compared to smaller animals. However, these challenges can be mitigated by using suitable alternative laboratory-based small animal models such as embryonated chicken eggs (ECE) or rodents. For example, Kurogi et al. (1978) employed a murine model to compare the pathogenicity of different strains of AKAV in mice. Chicken embryos infected with AKAV show lesions typically observed in natural AKAV infection in ruminants such as malformations of the legs and toes, arthrogryposis, dwarfism, cerebellar hypoplasia and hydranencephaly (Ikeda and Yonaiyama, 1978; Konno et al., 1988; McPhee et al., 1984).

As SBV is a relatively newly discovered virus the number of experimental infection studies is limited. Moreover, experimental infection with SBV in cattle and sheep during the most susceptible stages of gestation appears to only lead to foetal malformations in a very limited number of cases (EFSA, 2014) thus limiting the potential to investigate the pathogenesis of congenital Schmallenberg disease. Nevertheless, the use of laboratory-based small animal models, principally mouse models, has facilitated research on the biology and pathogenesis of this virus. Varela et al. (2013) demonstrated that SBV replicates in newborn NIH-Swiss mice and causes malacia and vacuolation of the cerebral cortex; lesions similar to those observed in aborted lambs and calves naturally infected with SBV. Type I interferon receptor knock-out (IFNAR-/-) mice are also susceptible to SBV infection and can develop fatal disease, suggesting that they may be a useful species for SBV vaccine research (Wernike et al., 2012).

However, a suitable small animal model is yet to be identified to study the pathogenesis of congenital SBV malformations in developing embryos. Considering the phylogenetic and epidemiologic similarities between SBV and AKAV and the teratogenic effects of AKAV in ECE models, it was hypothesised that chicken embryos might also provide a suitable small animal model to study SBV.

Hence, the aims of this research were, firstly, to investigate if chicken embryos are susceptible to experimental SBV infection and, if so, to what extent, and secondly, to evaluate the pathogenicity and teratogenicity of SBV and AKAV infection at a range of doses in an embryonated chicken egg model.

2. Material and methods

2.1. Study design

The study design and methodology, including the age at which embryos were inoculated and virus inoculum doses used were derived from previous studies which investigated the pathogenicity of Simbu serogroup Orthobunyaviruses in ECE models (Ikeda and Yonaiyama, 1978; Kitano et al., 1996, 1997; Konno et al., 1988; McPhee et al., 1984).

Two studies were conducted. In Study A, 0.2 mL of undiluted cell culture-grown SBV ($10^{6.4}$ TCID$_{50}$/0.2 mL) was inoculated into the yolk sac of chicken embryos at 6-days (n = 43) and 8-days (n = 41) of incubation. Undiluted cell culture grown SBV was inoculated at a high dose to maximise the potential for virus replication and pathology to occur. Control embryos were inoculated with 0.2 mL of phosphate buffered saline (PBS), pH 7.3, at 6-days (n = 40) and 8-days (n = 40) of incubation.

In Study B, SBV and AKAV were inoculated at a range of doses in order to compare pathogenicity and teratogenicity and secondly to evaluate if virus replication and induced congenital malformations were virus dose-dependent. To achieve this groups of approximately 40 embryos were infected with SBV (n = 178) or AKAV (n = 177) at virus doses ranging between $10^{2.0}$ and $10^{6.0}$ TCID$_{50}$/0.2 mL at 7-days of incubation. AKAV was not inoculated at $10^{6.0}$ TCID$_{50}$. Control embryos (SBV controls: n = 36; AKAV controls: n = 44) were inoculated with

0.2 mL of PBS at 7-days of incubation.

In both Study A and Study B, ECE were incubated at 37 °C until day 19 of incubation.

2.2. Pathological and virological examination

On day 19 of incubation ECE were euthanised (chilled at 4 °C for a minimum of 8 h) and submitted for necropsy examination. Embryos that died between day 7 and day 18 of incubation were also necropsied. Between day 14 and day 18 of incubation, five control embryos were harvested and submitted for necropsy examination daily for comparative pathology with embryos that had died. At necropsy examination, virus-inoculated embryos were examined and compared directly with the harvested control embryos of the same age; the musculoskeletal system and central nervous system were examined for evidence of macroscopic congenital defects or other pathological changes and recorded accordingly.

Sterile plain swabs of brain tissue were collected from a sub-sample of 19 day old ECEs (both virus-inoculated and control embryos) at necropsy and placed in 3 mL of phosphate buffered gelatin saline (PBGS) and held frozen at approximately −80 °C until tested for either SBV or AKAV RNA using quantitative real-time reverse transcription PCR (qRT-PCR).

2.3. Viruses

SBV (BH-80/11-4) used in this study was originally isolated at the Friedrich-Loeffler-Institute (Insel Riems, Germany) in 2011. The virus was initially isolated from bovine serum and passaged once in KC cells, five times in BHK$_{21}$ cells and, at the Elizabeth MacArthur Agriculture Institute (NSW, Australia), twice in hamster lung (HmLu-1) cells. Virus stocks were produced at the Elizabeth MacArthur Agriculture Institute in Hmlu-1 cells and stored at approximately −80 °C.

AKAV (strain B7949) was originally isolated in Australia in 1968 from *Culicoides* brevitarsis and passaged 3 times in suckling mouse brain, 9 times in vero cells and 3 times in HmLu-1 cells.

2.4. Virus titration

Virus titres were determined by endpoint titration in HmLu-1 cells and expressed as tissue culture infective doses at a 50% endpoint (TCID$_{50}$) (Reed and Muench, 1938).

2.5. Virus inoculation

Virus inocula were prepared using serial 10-fold dilutions of stock virus in sterile PBS. Chicken embryos were inoculated by the yolk sac route.

2.6. Embryonated chicken eggs (ECE)

Embryonated chicken eggs were sourced from a commercial hatchery (Multiquip, Maldon, NSW, Australia) at 6, 7 and 8-days of incubation.

2.7. Quantitative real-time PCR

PBGS-containing swabs of brain tissue were tested for SBV RNA using qRT-PCR adapted from the method previously described (Bilk et al., 2012; Hoffmann et al., 2012) and for AKAV RNA using an unpublished assay (Gu and Kirkland, personal communication).

In brief, nucleic acids were extracted from 50 μL of the PBGS solution (which the swab of brain tissue was stored in at −80 °C pending analysis) using the MagMax-96™ Viral RNA Isolation system (Ambion, TX, USA) in accordance with manufacturer's guidelines on a Kingfisher-96 magnetic particle handling system (Thermo, Finland) using the