Review of Ebola Virus Infections in Domestic Animals

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Abstract: Ebola viruses (EBOV; genus Ebolavirus, family Filoviridae) cause often fatal, hemorrhagic fever in several species of simian primates including human. While fruit bats are considered a natural reservoir, the involvement of other species in the EBOV transmission cycle is unclear, especially for domesticated animals. Dogs and pigs are so far the only domestic animals identified as species that can be infected with EBOV. In 2009 Reston-EBOV was the first EBOV reported to infect swine with indicated transmission to humans; and a survey in Gabon found over 30% seroprevalence for EBOV in dogs during the Ebola outbreak in 2001-2002. While infections in dogs appear to be asymptomatic, pigs experimentally infected with EBOV can develop clinical disease, depending on the virus species and possibly the age of the infected animals. In the experimental settings, pigs can transmit Zaire-Ebola virus to naive pigs and macaques; however, their role during Ebola outbreaks in Africa needs to be clarified. Attempts at virus and antibody detection require as a prerequisite validation of viral RNA and antibody detection methods especially for pigs, as well as the development of a sampling strategy. Significant issues about disease development remain to be resolved for EBOV. Evaluation of current human vaccine candidates or development of veterinary vaccines de novo for EBOV might need to be considered, especially if pigs or dogs are implicated in the transmission of an African species of EBOV to humans.
**INTRODUCTION**

Ebola viruses (EBOV) are enveloped, single-stranded, negative sense RNA viruses classified into the family Filoviridae. The African species of EBOV, Zaire, Sudan and Bundibugyo ebolaviruses cause severe haemorrhagic fevers in humans, with high fatality rates. *Cote d’Ivoire ebolavirus* is considered to be less virulent in humans, while *Reston ebolavirus*, the only species found outside Africa, has never been associated with human disease, despite documented exposures [1-3].

Accumulated evidence has confirmed fruit bats as the natural reservoir of EBOV [4, 5], while several wildlife species have been identified as susceptible to infection with EBOV; and bushmeat has been implicated in the initiation of some human outbreaks. However, the role of domestic animals is not yet clear, and to date only limited sampling has been conducted during or shortly after Ebola outbreaks seeking to determine whether domestic animals can be infected with Ebola virus and possibly participate in the virus transmission cycle. From five tested species – bovine, ovine, caprine, swine and canine – only dogs were found to have antibodies against EBOV [6, 7]. Recently, Reston-Ebola virus (REBOV) has been isolated from pigs in the Philippines, and importantly with further transmission to humans, which raises a concern that this species may have been missed as susceptible to EBOV during the surveys due to the number of samples being limited to only twelve pigs.

**INFECTION OF SWINE WITH REBOV**

In 2008, REBOV was detected in pigs in the Philippines. The Philippine Department of Agriculture requested assistance from the US Department of Agriculture Foreign Animal Disease Diagnostic Laboratory (FADDL) and submitted samples for laboratory diagnostics, because of the suspected outbreak of highly pathogenic porcine reproductive and respiratory syndrome (PRRS) with high mortality on the affected swine farms. FADDL did indeed confirm infection with PRRSV, with some pigs also being co-infected with porcine circovirus 2. In addition, the laboratory detected an L gene of REBOV on the panviral microarray. Therefore, samples were transferred to CDC Atlanta; and the presence of REBOV was confirmed [8]. Subsequently, antibodies to the virus were found in several pig farmers, providing a strong indication that they had acquired the virus from infected pigs [8, 9].

Experimental inoculation of swine with REBOV did not reproduce the high mortality observed in the Philippines, suggesting that the mortality in swine was likely due to the highly pathogenic PRRSV. Marsh and co-authors infected oronasally eight 5-6 weeks old piglets with 10⁶ TCID₅₀ of the Philippines 2008 isolate of REBOV, and eight piglets of the same age subcutaneously with the same dose of virus [10]. Shedding from the nasopharynx at the RNA and infectious virus levels was detected in all pigs inoculated oronasally. The virus was detected in
multiple organs in all inoculated pigs, although the piglets remained clinically healthy [10].

**Infection of Swine with ZEBOV**

Infections of swine with the African species of *ebolavirus* have not been reported; however, in the experimental infection studies, *Zaire ebolavirus* (ZEBOV) appears to be far more pathogenic than REBOV in the same age group of pigs. Domestic pigs (Landrace breed) challenged by oronasal and ocular routes with a total of $1 \times 10^6$ PFU of ZEBOV developed severe respiratory distress requiring euthanasia for humane reasons. Under similar experimental conditions, virus transmission from infected to naive animals was evaluated in a second set of pigs. These piglets were one week younger than the first group, and interestingly developed only mild clinical signs, but transmitted the virus to co-housed piglets of the same age [11]. The two experiments were repeated, this time inoculating piglets from the same litter – six at the age of 4 weeks and six at the age of 6 weeks. The clinical outcomes were strikingly similar: younger piglets displayed mild to moderate clinical signs, while the older piglets again developed rather severe respiratory distress [12, 13]. Interestingly, the age difference in development of clinical disease may be related to higher levels of interferon-α at the time of inoculation with EBOV in the younger piglets (mean value of 60 pg/ml of serum compared to 30 pg/ml in the older piglets) (unpublished), an observation worth further investigation.

Transmission of ZEBOV from infected piglets was demonstrated not only to all naive pigs co-habitating with inoculated animals [11], but also to all cynomolgus macaques housed in an open inaccessible cage system in the same room as the infected piglets [12].

**Pathogenesis of EBOV in Swine**

Infection of swine with EBOV is systemic with major involvement of the respiratory tract as well as pathology in the lungs. The major route of virus shedding is from the nasopharynx, especially for infections acquired via the oronasal route. The severity of pathological changes and the development of clinical signs are likely to depend on the infecting species of *ebolavirus*, as well as the age, and possibly also on the genetic background of the pigs.

Following mucosal exposure, pigs replicated ZEBOV to high titers (reaching $10^7$ TCID$_{50}$/ml), mainly in the respiratory tract, and also developed severe lung pathology. Oronasal shedding was detected for up to 14 days post-infection (dpi); and the virus was also briefly detected in blood. Neutralizing antibodies were detected only at around 10 dpi, while the first IgM antibody detection by ELISA occurred between 5 and 7 dpi [11,13]. Oronasal shedding was confirmed for REBOV infected piglets, but the lung pathology was considerably less severe; and
the virus was not detected in the blood. IgG antibody development was detected using ELISA in subcutaneously inoculated piglets at 8 dpi, when only 2 out of 4 oronasally infected pigs seroconverted. All pigs were considered positive for EBOV IgG antibody at 10 dpi [10], similar to the antibody development against ZEBOV.

In addition to lung and nasal turbinates, the infectious virus or viral RNA of either ZEBOV or REBOV was detected in the liver, spleen, tonsil, bronchiolar and submandibular lymph nodes, and at low levels in the heart, kidney and intestine. REBOV RNA was also detected in skeletal muscle, but the RNA detection attempts in the muscle of ZEBOV infected piglets were negative [10, 11].

Although alveolar macrophages appear to be the most important target for EBOV in the lungs of both piglets and non-human primates, the observed outcome of the infection of macrophages in terms of histopathological lesions in the lungs is notably different. Almost no infiltration of other immune cells into the lungs was observed for macaques infected with ZEBOV [12] or pigs infected with REBOV [10], while massive infiltration of not only macrophages but also neutrophils and lymphocytes was observed in the lungs of older piglets infected with ZEBOV, although antigen was present only in the alveolar macrophages. The infiltration had essentially reached the point when no alveolar space was left, and lead to necrosis 5-7 days post-infection [11]. Microarray analysis of changes in gene expression in lung tissue from the older ZEBOV-infected piglets detected upregulation of a high number of proinflammatory cytokines, chemokines and acute phase protein genes, known to attract immune cells to sites of infection, essentially resulting in a cytokine storm [13]. The detected upregulation of the anti-inflammatory cytokine IL-10 genes in ZEBOV-infected lungs did not appear to be sufficient to control the inflammatory process. An overactivation of the pulmonary proinflammatory response likely plays a crucial role in the pathogenesis of ZEBOV infection in pigs through an out of control attraction of inflammatory cells to the lungs in the experimentally inoculated piglets older than 5 weeks.

Systemic effects included a decline in the proportion of monocyte/dendritic and B cells in peripheral blood [13], which further contributed to the disregulation of the adaptive immune response noticeable because of a somewhat delayed production of antibodies, with the IgG class antibodies and the virus neutralizing antibodies detected only about two weeks post-infection [10, 11], as described above.

**Diagnostic Assays**

A critical aspect of reliable diagnostics is a well-designed approach to sample collection (Fig. 1). Since REBOV could not be detected in the blood of infected pigs, nasopharyngeal swabs collected between 4 - 8 days post infection might be the only live sampling available for virus detection. This leaves a period of about a week when infection with REBOV would not be detected, until the development of antibodies around 2 weeks post-infection [10]. From a diagnostic point of view, the situation with ZEBOV is better than with REBOV, as shedding ceases with the
Fig. 1. Overview of current understanding of disease development and sampling strategies for virus detection in ZEBOV-infected swine.
development of antibodies; and virus RNA is detectable in the blood, at least briefly, around 5 days post-infection [11, 13]. However, the duration of detectable levels of antibodies in pigs is not known.

**Antigen detection**

Antigen detection by immunohistochemistry in fixed tissues, especially lungs and lymph nodes using rabbit or mouse polyclonal anti-EBOV antibodies appears to be reliable [8, 10, 11]. An approach using monoclonal antibodies might need to be considered for consistent long-term testing.

**Viral RNA detection**

Viral RNA detection was performed using different real-time RT-PCR assays targeting the L gene [12], the glycoprotein gene [11] or the nucleoprotein gene [10]. All these approaches were sensitive in swine samples, as well as comparable between laboratories where applicable, e.g. between the National Microbiology Laboratory (NML) and the National Centre for Foreign Animal Disease (NCFAD), with differences laying more in respective biosafety protocols as well as in different instrumentation between the laboratories. Harmonization may be required, when necessary.

**Virus isolation**

Vero E6 cells are currently the preferred cell substrate for EBOV isolations. Although virus stock preparation did not show any difference between Vero E6 cells from NML compared to NCFAD cells, EBOV isolation from swine swabs and tissues did indicate differences in the formation of the cytopathic effect (CPE) between the two laboratories. EBOV re-isolated from swine did replicate, based on progressively increasing genomic copy number during the passage of the isolates, but did not cause CPE in the NCFAD Vero E6 cells until the second or third passage. Therefore, blind passages during isolation attempts from field samples might be required. Currently, swine kidney cell line (PK15) is under evaluation as a candidate for virus isolation from swine samples. The classical CPE-reduction virus neutralization assays (detection of neutralizing antibodies in serum) in Vero E6 cells are suitable for swine serum samples.

**Antibody detection**

Antibody detection by ELISA employing cell lysate antigen [14] was successfully used in the serosurvey for EBOV antibodies in dogs [7]. This ELISA format, using CDC antigen, was used in IgM capture assay in pig serum by Nfon et al [13], as well as in the study by Marsh and colleagues [10] to detect IgG antibodies in their experimentally infected piglets. IgG detection using ELISA employing the inactivated purified whole virions as an antigen was also successfully used for swine serum [11]. ELISAs for antibody detection in swine sera will likely require regional validation to determine diagnostic specificity in swine. An understanding of the
dynamics of EBOV antibody development in pigs would be important; especially if use of recombinant antigens would be considered in designing ELISA assays for lower containment laboratories.

At the present time, there are many unanswered questions about disease development caused by EBOV in swine. For example: the extent to which the development of different Ebola virus infections is linked to the age of the infected animal or the extent to which the virus can be shed without apparent disease. Despite the growing body of knowledge about the nature of Ebola viruses, it remains uncertain whether the transmission of these viruses can be sustained in a swine herd. Even more disturbing is a further unanswered question: Can the evolution or adaptation of REBOV in swine lead to mutations which would allow for the virus to be pathogenic in humans? In view of the fact that viruses can be present in the semen of other species, the possibility that artificial insemination might lead to extensive EBOV transmission among swine needs to be investigated.

**CONCLUSION**

The experimental studies cited above have confirmed that pigs infected with REBOV shed the virus without clinical signs; and this shedding may also apply to some pigs infected with ZEBOV [10, 12]. In addition, clinical signs of Ebola virus infections are not specific and resemble other infections of the respiratory system, e.g. PRRSV. Infected animals pose a transmission risk to humans [10,12]. The Food and Agricultural Organization (FAO) pig density map for Uganda (http://geonetworkfao.org) shows that outbreaks of Ebola virus have taken place in areas of great pig density, raising concern about potential of ZEBOV epidemics due to transmission of the virus to pigs, and subsequently to humans. It is now evident that the infection of an index human case would be sufficient to introduce the Ebola virus into the human population, the evaluation of current human vaccine candidates or the development of veterinary vaccines for EBOV de novo might need to be considered as well as adaptation and evaluation of existing diagnostic tests for veterinary purposes.

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