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Research article

Investigating African swine fever virus susceptibility across seven genera of peccaries and pigs using peripheral blood mononuclear cells

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Abstract

African swine fever (ASF), a viral haemorrhagic fever of suids, has recently transformed from an exotic disease to a panzootic threat to domestic and wild suids worldwide. By 2023, ASF had reached large parts of Europe and Asia, parts of the Americas, and with the Democratic Republic of Timor-Leste the doorstep of Australia. The disease is caused by a large and very complex DNA virus of the genus *Asfivirus* in the family *Asfarviridae* that replicates primarily in monocytes and macrophages. So far, susceptibility to ASF virus (ASFV) has only been shown for members of the Suidae family. With regard to members of the Tayassuidae family, only collared peccaries *Dicotyles tajacu* have been investigated and showed no obvious susceptibility. In the present study, the susceptibility of peccaries was further investigated using blood-derived monocytic cells from a collared peccary, a Chacoan peccary *Catagonus wagneri* and a white-lipped peccary *Tayassu pecari* from European zoos. Viral replication was monitored using indirect immunofluorescence staining and hemadsorption tests. Unlike cells from Suidae including domestic pigs *Sus scrofa domesticus*, wild boar *S. scrofa*, bearded pig *S. barbatus*, Visayan warty pig *S. cebifrons,* Sulawesi babirusa *Babyrousa celebensis*, red river hog *Potamochoerus porcus* and warthog *Phacochoerus africanus*, Tayassuidae macrophages did not support ASFV replication and thus susceptibility is highly unlikely. There is no evidence that peccaries could play any role in ASF epidemiology.

Introduction

African swine fever (ASF) is considered one of the most complex viral diseases affecting both domestic and wild pigs. In domestic pigs *Sus scrofa domesticus* and Eurasian wild boar *S. scrofa*, the disease is highly lethal and exhibits similarities to a viral haemorrhagic fever (Pikalo et al. 2021). Having originated in Africa, ASF has spread to diverse regions of the world, including Europe, Asia and even sections of the Americas, which has earned ASF the nickname of the 'forgotten pandemic' (Ward et al. 2021). Beyond its sylvatic cycle between soft ticks of the genus *Ornithodoros* and common warthogs *Phacochoerus africanus* in Africa, the infection is primarily transmitted through direct contact with infected pigs or via contaminated items such as vehicles, tools, feed (Chenais et al. 2018) or pork products produced from infected animals. Although not posing a direct risk to humans, the impact of ASF can have devastating consequences for the pork industry (Dixon et al. 2020; Sánchez-Vizcaíno et al. 2015), food security of humans (Meijaard et al. 2024), ecosystems inhabited by wild suids (Luskin et al. 2021) and threatens all eleven endemic

wild pig species in Southeast Asia, such as Endangered Oliver's warty pigs *Sus oliveri* and Vulnerable bearded pigs *Sus barbatus* (Luskin et al. 2021). In addition, ASF is a threat to endangered wild pigs like Vulnerable Sulawesi babirusa *Babyrousa celebensis* and Critically Endangered Visayan warty pigs *Sus cebifrons* kept in zoos. Specifically, Eurasian wild boar can serve as a reservoir and may perpetuate the spread of the disease in ecosystems (Chenais et al. 2018). Being descendants of domestic pigs, wild boar or hybrids of both, this extends to feral pigs especially in the Americas and Australia.

The influence of ASF on wildlife goes beyond mere direct mortality. Infected pigs have the potential to act as mechanical vectors, disseminating the virus to other susceptible populations within their environment. The absence of wild pigs as prey can also have repercussions on endangered carnivores like tigers *Panthera tigris* (Luskin et al. 2023). Additionally, measures taken to manage ASF such as eliminating infected populations or enforcing stringent biosecurity protocols may disturb ecosystems and result in unintended effects on biodiversity. The consequences of ASF outbreaks can directly and indirectly impact animals under human care including those in zoos. For instance, cases of the disease in the wild boar population or domestic pigs may prompt extended periods of confinement, transport bans or even culling of zoo stock and pose a direct risk to threatened species. This not only has welfare implications, but hinders important conservation breeding of highly threatened species (e.g. Visayan warty pig).

Given that ASF currently threatens all wild pig species endemic to Southeast Asia (Luskin et al. 2021), discussions on susceptibility have accelerated and this further highlights the necessity to adapt control methods for both animals in human care and wild animals. This extends to the Tayassuidae (peccaries) which inhabit South and Central America and partially the southwestern part of North America. Despite belonging to a distinct taxonomic family, peccaries share similarities in physiognomy, physiology and habitat with true pigs. As a consequence of these similarities, discussions regarding the cross-species transmission of ASF virus to peccaries are ongoing. So far, scientific evidence suggesting that peccaries can be infected with ASF virus (ASFV) is scarce. Recognising the significant ecological and economic impacts of ASF, further research on peccary susceptibility and other pig species is essential for a comprehensive understanding of ASF transmission, its implications for wildlife populations as well as populations of (threatened) pigs and peccaries kept under human care. Conservation breeding programmes in Europe, Asia and North America for the Endangered Chacoan peccary could be directly and indirectly affected by ASF incursions.

In this study, an attempt to gain first insights into the susceptibility of various Suidae and Tayassuidae species to ASFV was made. Utilising macrophages derived from peripheral blood mononuclear cells (PBMCs) of different species, susceptibility was assessed. The samples were obtained during routine hygiene inspections or culling in different zoos in Germany as well as from animal experiments at the Friedrich-Loeffler-Institut Riems (FLI), Germany.

Materials and methods

Sample origin

Whole blood samples of pig species were acquired from zoos during routine health/hygiene examinations or culling. All samples were transported to FLI Riems immediately after sample collection to ensure optimal viability of leukocytes. Samples of Eurasian wild boar, minipig, domestic pig, red river hog (originating from Cologne Zoo) and warthog (from the zoo in Magdeburg) were obtained from individuals included in animal trials at FLI. These trials were performed in accordance with the most recent German animal welfare regulations and approved by local authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern [LALLF M-V]) under reference 7221.3-2-023/15 (minipig) and 7221.3-1-034/23 (Eurasian wild boar, domestic pig, red river hog and warthog). Table 1 provides an overview of all samples that were used to generate the results presented in this study.

Experimental setup and viruses

For isolation of PBMCs, whole ethylenediaminetetraacetate (EDTA)-blood was mixed with Hanks dextran (10% solution) at a ratio of 1:10. After an incubation of 90 min at room temperature,

Table 1. Summary of samples used in this study. Indicated are number of samples per species and their origin.

Species	# of samples [n]	Origin/zoo
Chacoan peccary *Catagonus wagneri*	3	Berlin
Collared peccary *Pecari tajacu*	3	Nordhorn
White-lipped peccary *Tayassu pecari*	1	Berlin
Visayan warty pig *Sus cebifrons*	2	Berlin
Bearded pig *Sus barbatus*	1	Berlin
Sulawesi babirusa *Babyrousa celebensis*	1	Kronberg
Red river hog *Potomochoerus porcus*	3	Cologne
Warthog *Phacochoerus africanus*	2	Magdeburg
Eurasian wild boar *Sus scrofa*	6	Riems
Domestic pig *Sus scrofa domesticus*	6	Riems
Minipig *Sus scrofa domesticus*	6	Riems

the PBMC-containing supernatant was collected and washed with 1X phosphate-buffered saline (PBS, Gibco) before seeding in Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 10% fetal calf serum and 0.01% penicillin/streptomycin, Gibco). Where hemadsorption tests (HAT) were carried out, the fraction containing red blood cells (RBCs) was collected and diluted in 1X PBS at a ratio of 1:10. Cells were seeded into 96-well plates at a density of ~3×10^5 cells/well and incubated at 37°C in the presence of CO^2 and a humidified atmosphere. After 24 h of incubation, pig recombinant colony-stimulating factor 2 (GM-CSF) was added at a concentration of 2 ng/ml to initiate differentiation of monocytes. Following a differentiation period of 30 h, cells were inoculated with ASFV (100 µl/well). Since high-quality RBCs are essential for HAT performance, the level of RBC osmotic stress was evaluated upon arrival of the samples. If the proportion of RBCs under osmotic stress was low (isotonic), HAT was conducted by inoculating GM-CSF differentiating cells with ASFV field strains *'Armenia08'* ($10^{6.25}$ HAD_{50}) or *'Prenzlau22'* (10^6 HAD_{50}). The former virus was isolated from an outbreak among domestic pigs in Armenia in 2008. The latter originates from an outbreak in domestic pigs in Germany in 2022. After 24 h, donor-specific RBCs were added to each well at a ratio of 1:40, allowing rosette formation. Results were analysed after 24 h and 48 h using a brightfield microscope.

If a significant number of RBCs exhibited osmotic stress, whether hypertonic or hypotonic, the cells were infected with a genetically modified strain of ASFV *'Armenia08'* (ASFV Armenia Δ248LGFPhuCD4). This strain, capable of replicating in both macrophages and permanent wild boar lung cells, was generated through homologous recombination. Specifically, a transfer plasmid was used to delete ASFV gene 248K and insert a green fluorescent protein (GFP) under the control of the ASFV p72 promoter, as well as a truncated version of the human CD4 cell surface protein under the control of the ASFV p30 promoter (Hübner et al. 2019). After an incubation of 72 h, the ASFV-specific GFP signal was acquired using a Thunder Imager (Leica). To ensure accuracy of negative results (when no ASFV infection could be observed), classical swine fever virus (CSFV) infection was used to demonstrate the presence of susceptible cells. Here, PBMCs of a Chacoan peccary, a white-lipped peccary and a domestic pig were either infected with ASFV *'Armenia08'* ($10^{6.25}$ HAD_{50}) or CSFV *'Rösrath'* at a titer of 10^3 tissue culture infectious dose (TCID). The amount of ASFV- and CSFV-infected cells was assessed by staining nuclei with Hoechst 33342 (Thermo Fisher Scientific) and infected cells with a polyclonal rabbit α-p72 antibody (ASFV) or a monoclonal mouse α-E2 antibody mix (CSFV). Both primary antibodies were stained with an Alexa-488-labelled secondary antibody (goat-α-mouse-IgG or donkey-α-rabbit-IgG, Thermo Fisher Scientific). All nuclei and ASFV/CSFV signals were counted using ImageJ to calculate the percentage of infected cells in each well. Furthermore, since publications in the ASFV field indicate that differentiation of blood-derived monocytes with M-CSF for three days prior to infection with ASFV may be needed for optimal infection rates in vitro (McCullough et al. 1999), PBMCs of a Chacoan peccary, a white-lipped peccary and a domestic pig were incubated as controls with either human M-CSF, pig GM-CSF or no supplement for 30 h or 72 h prior to ASFV/CSFV infection to verify the protocol suitability for assessing general susceptibility to ASFV.

Results

Overall, the viability of cells isolated from all blood samples ranged from 78–98%, rendering them suitable for subsequent experiments. Examination of whole blood revealed that ~50% of all RBCs in samples of the babirusa and the bearded pig were under osmotic stress, while all other samples exhibited negligible osmotic stress in RBCs. The following HATs yielded ASFV-positive results for the Visayan warty pig, domestic pig, Eurasian wild boar, red river hog, warthog and minipig (Figure 1). In contrast, no rosette formation was observed in samples from Chacoan peccary, white-lipped peccary and collared peccary. Fluorescence revealed that GM-CSF-treated monocytes derived from babirusa and bearded pigs were susceptible to ASFV infection, as indicated by the presence of GFP-signal in the cytoplasm of cells (Figure 2). The Visayan warty pig served as a positive reference in fluorescence experiments.

Since no rosette formation was observed for any peccary species tested, these results were validated further. Cells from a Chacoan peccary, a white-lipped peccary and a domestic pig were infected with either ASFV *'Armenia08*' or CSFV *'Rösrath'*. As CSFV and ASFV largely target the same cells for infection, CSFV infection was used as a control to verify the general presence of suitable target cells for ASFV. In accordance with results from HAT, no ASFV-infected cells were observed in the peccary samples (Figure 3). The domestic pig on the other hand displayed up to 80% ASFV-infected cells. Additionally, no significant difference in the number of ASFV-infected cells was observed when comparing cells incubated with M-CSF, GM-CSF or no supplement for 30 h. After infection with CSFV, infection was observed in both the domestic pig and the peccary species. Cells of the domestic pig showed up to 74% infected cells, while the peccary species showed up to 53%. The choice of supplement had no significant effect on the percentage of CSFV-infected cells. Increasing the incubation time prior to infection to 72 h rendered comparable results and no ASFV signal in peccary cells (results not shown).

Discussion

African swine fever continues to spread, threatening more and more endemic species of wild pigs in Southeast Asia and therefore whole ecosystems (Luskin et al. 2021). The regions not yet affected include large parts of the American continent where only the island of Hispaniola has been affected so far (Ruiz-Saenz et al. 2022) and Australia where the virus is already close (Shaw et al. 2024). If the disease were to become established in South and Central America and Australia, it would encounter new ecosystems with pigs and peccaries. The feral pig populations in the Americas and Australia are descendent from domestic pigs and thus as susceptible as domestic pigs and Eurasian wild boars and will pose similar challenges to control (Brown and Bevins 2018; Shaw et al. 2024). However, there is little data on the three peccary species, two of which are listed as endangered. Their involvement in ASF epidemiology may be critical to the survival of the species. This also applies if peccaries are not susceptible to ASFV and cannot spread the virus, because they might be affected by disease management actions such as trapping and culling.

The present study attempted to assess the risk of susceptibility to ASFV in peccaries in general and in Asian pig species thought to be susceptible mainly because they belong to the Suidae family.

A study based only on primary cell cultures can only be indicative as the whole organism is necessary for the outcome of a possible infection. Monocytes and macrophages from red river hogs and warthogs are certainly susceptible to infection but the animals do not develop clinical signs (Jori and Bastos 2009; Jori et al. 2013). The situation is different in domestic pigs, minipigs and Eurasian wild boar. These species belong to the genus *Sus* and show the full clinical picture of ASF when infected with highly virulent strains, with all the signs of viral haemorrhagic fever resulting in the death of animals within four to seven days after the onset of clinical signs (Blome et al. 2020).

However, the complete lack of susceptibility at the cell level is a very strong indication that peccaries are not susceptible to ASFV. Additionally, by conducting in vitro infection with CSFV, it

was shown that the target cells necessary to establish a successful ASFV infection were present. Previous studies could demonstrate via detection of CSFV-targeting antibodies in Colombian collared peccaries that peccaries are susceptible to CSFV infection (Montenegro et al. 2018). Taken together, this suggests the existence of family-specific factors e.g. differences in cellular receptors or intracellular antiviral mechanisms that limit ASF virus entry or replication within peccary monocytes and macrophages. Here, further investigation into the molecular and cellular mechanisms underlying this resistance could provide valuable insights into ASF pathogenesis and host-virus interactions.

The lack of virus replication in peccary cells is consistent with the only published in-vivo study on ASF susceptibility in collared peccaries (and therefore just one out of three genera and species

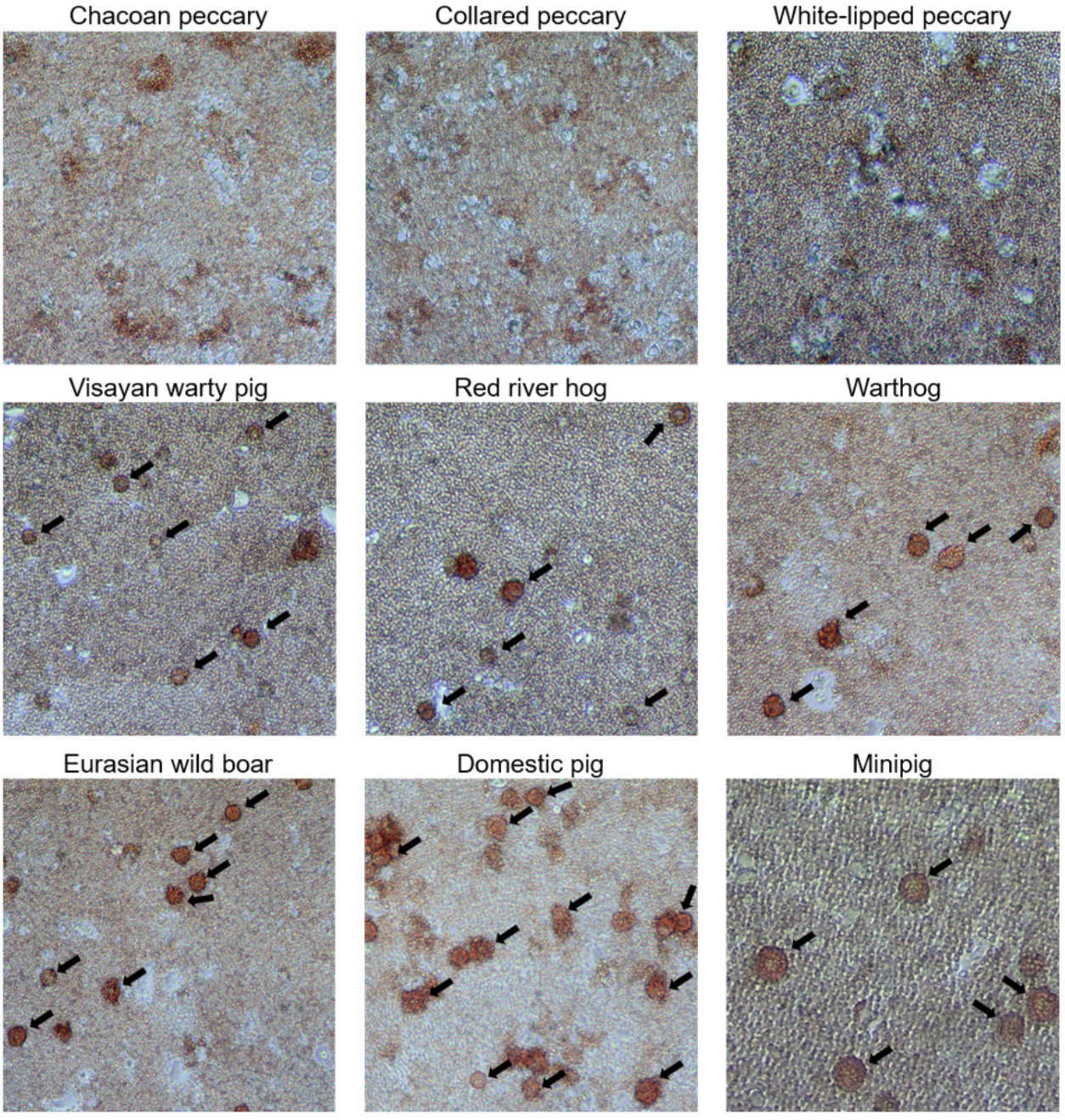

Figure 1. Hemadsorption tests revealed varying susceptibility of monocytes and macrophages derived from different pig and peccary species. Cells were inoculated with African swine fever virus (ASFV) field strains '*Armenia08*' and *'Prenzlau22'* after pig recombinant colony-stimulating factor 2 (GM-CSF) treatment. Addition of donor-specific red blood cells results in rosette formation around ASFV-infected cells. Uninfected cells remain without rosettes. Images are representative of all individuals per species tested.

of peccaries) by Dardiri et al. (1969). Based on the current results, the lack of susceptibility seems to apply to all peccary species belonging to three different genera. This is in contrast to pigs in which different genera show different susceptibility to ASFV. This has implications for measures to be taken in the wild as well as in zoos and other facilities where peccaries are kept following an ASF outbreak. Quarantine, stabling orders or even pre-emptive culling are not necessary. Especially in the Endangered Chacoan peccary, culling of wild specimens as a supposed ASF management measure would be fatal, because the Dry Chaco as natural habitat of this endemic species has one of the highest deforestation rates in the world. According to Camino et al. (2022) there will be no habitat for Chacoan peccaries left by 2051 outside of protected areas if the deforestation is not stopped.

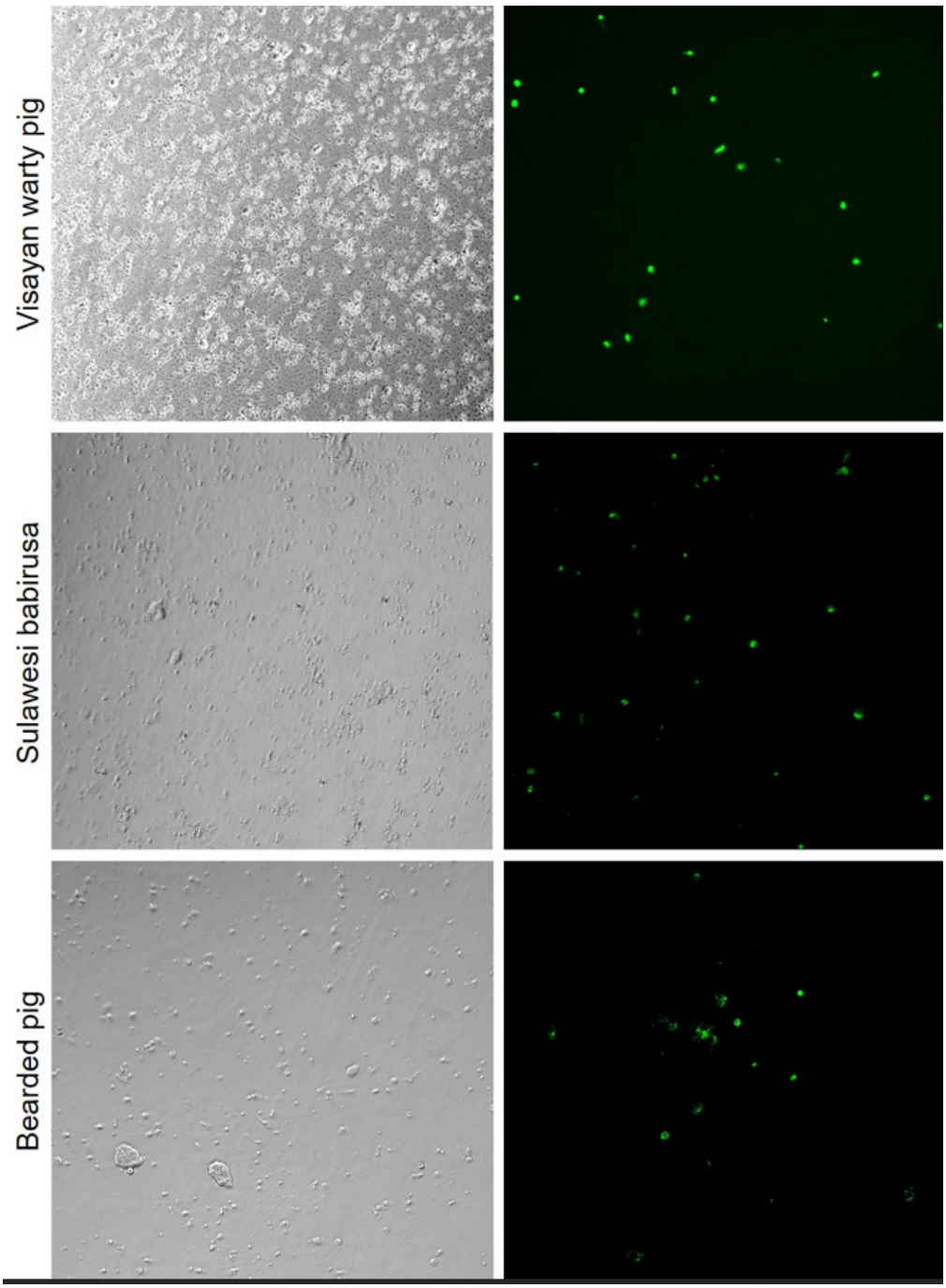

Figure 2. Detection of green fluorescent protein (GFP)-expressing African swine fever virus (ASFV) demonstrates susceptibility of Asian wild suids. Isolated peripheral blood mononuclear cells (PBMCs) were treated with pig recombinant colony-stimulating factor 2 (GM-CSF) to initiate differentiation of monocytes. Cells were inoculated with Δ248L GFP '*Armenia08*', resulting in fluorescence of successfully infected cells. Depicted are representative wells in bright field (left column) and respective 488 nm channel to assess GFP signal (right column).

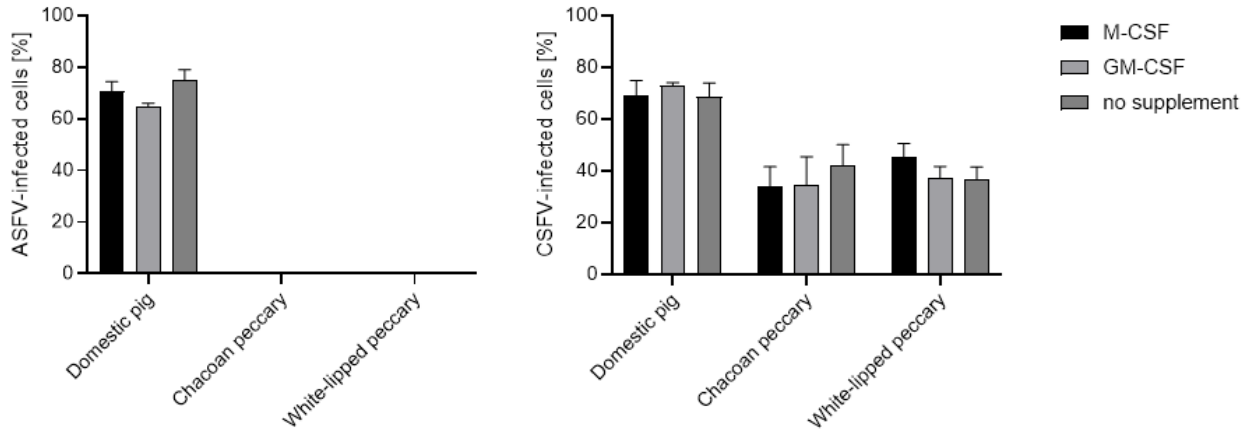

Figure 3. Verification of insusceptibility of peccary cells to African swine fever virus (ASFV) infection in vitro. Cells of various peccary species and a domestic pig as control were incubated with either M-CSF, GM-CSF or no supplement prior to infection with ASFV (left) or classical swine fever virus (CSFV) (right). The percentage of infected cells was calculated by counting stained nuclei and ASFV- or CSFV-specific immunofluorescence. Bars represent mean percentage of four technical replicates±SD.

In contrast, monocytes and macrophages from Suidae species were susceptible, as expected. This was also true for babirusa, warty pigs and bearded pigs. These results are consistent with the outbreaks observed for example on Negros Island and Borneo (Ewers et al. 2021). Unfortunately, all threatened species are fully susceptible with consequences for their survival in case of ASF incursions. Hope could come from oral vaccination which is currently under investigation e.g. in EU funded research projects. These vaccines would have to be integrated into adapted control measures and would only present one tool for ASF control but could become a game changer in fighting ASF in wild pig conservation (Muñoz-Pérez et al. 2021). To foster vaccine design, studies into basic mechanisms of susceptibility and beneficial host responses are currently carried out using African wild suids in collaboration with zoos and research institutions (Beckmann et al. 2024).

Conclusion

This study demonstrates that the susceptibility of monocytes and macrophages to ASFV infection in vitro varies between different peccary and pig species. While cells from peccaries were not susceptible at any point or setting, all members of the Suidae family showed susceptibility as expected. In addition, this study highlights the value of zoo-housed animals for research and conservation, even of non-threatened species like red river hogs, warthogs and collared peccaries.

Based on our studies and published in-vivo data, peccaries will most likely not play a role in ASF transmission and maintenance. Given the lack of evidence for successful infection (virus or antibody detection), excluding peccaries from all measures executed in ASF management in situ and ex situ should be considered.

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