Serological Evidence for the Circulation of Ebolaviruses in Pigs From Sierra Leone

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Many human ebolavirus outbreaks have been linked to contact with wildlife including nonhuman primates and bats, which are assumed to serve as host species. However, it is largely unknown to what extent other animal species, particularly livestock, are involved in the transmission cycle or act as additional hosts for filoviruses. Pigs were identified as a susceptible host for Reston virus with subsequent transmission to humans reported in the Philippines. To date, there is no evidence of natural Ebola virus (EBOV) infection in pigs, although pigs were shown to be susceptible to EBOV infection under experimental settings. To investigate the potential role of pigs in the ecology of EBOV, we analyzed 400 porcine serum samples from Sierra Leone for the presence of ebolavirus-specific antibodies. Three samples reacted with ebolavirus nucleoproteins but had no neutralizing antibodies. Our results (1) suggest the circulation of ebolaviruses in swine in Sierra Leone that are antigenically related but not identical to EBOV and (2) could represent undiscovered ebolaviruses with unknown pathogenic and/or zoonotic potential.

Keywords. Ebola; ELISA; pigs; neutralization test; West Africa.

Infections with Ebola virus (EBOV), the most prominent representative in the genus Ebolavirus (family Filoviridae), mostly lead to severe disease outbreaks in humans and nonhuman primates with fatality rates up to 90% [1]. Ebolaviruses have been studied for more than 4 decades since their first discovery in 1976 [2, 3]. However, our understanding of the virus ecology is rather limited, and the natural reservoir host species remains to be definitively clarified. There is molecular evidence for EBOV circulating in wildlife such as great apes (chimpanzees [Pan troglodytes], gorillas [Gorilla gorilla]) and duikers (Cephalophus spp) [4]. Although those animals are widely suspected to be incidental hosts, certain fruit bat species have been considered to be the most likely natural reservoir host of EBOV based on serological and rare molecular evidence [5–7]. Besides EBOV (Zaire ebolavirus species), there are currently 4 other known ebolavirus species classified with reported circulation in Africa and Asia and with varying human pathogenic potential. The isolation of Reston virus (RESTV) from pigs in the Philippines and RESTV-specific antibodies found in healthy farm workers in contact with the infected pigs [8] have clearly broadened our current understanding of the geographical distribution, human pathogenicity, and host range of ebolaviruses, suggesting that species other than bats can also play a role in ebolavirus ecology.

Experimental infection of pigs with EBOV determined predilection of the virus for the respiratory tract with oronasal shedding and subsequent transmission to cohoused piglets and to nonhuman primates without a direct contact [9, 10]. In addition, epidemiological data from EBOV disease (EVD) outbreaks in Uganda was associated with periods of peak pork consumption [11]. Although these peaks mainly coincided with the most important public holidays that might in turn contribute to the emergence of an EVD outbreak by big family gatherings and higher travel activities, this study emphasized the need to further investigate the potential role of pigs in ebolavirus maintenance and transmission cycle.

To date, the serological status of pigs in known endemic areas is mostly unknown, whereas the majority of past and ongoing serosurveillance studies have mainly focused on wildlife, such as bats [5–7], as well as on humans [12–15]. In this study, we collected 400 porcine sera from 3 different districts in Sierra Leone in 2016 to serologically investigate the potential role of pigs in ebolavirus ecology focusing on ebolavirus species and not testing for cross-reactivity against proteins from other filoviruses such as marburgviruses. The sera were initially tested in an in-house enzyme-linked immunosorbent assay (ELISA), with confirmatory testing by immunoblot analysis using individually expressed viral proteins as well as virus-like particle (VLP) preparations. Sera that were reactive in those assays were further analyzed in a newly established serum neutralization...
test based on transcription and replication competent VLPs (trVLPs) under biosafety level (BSL)2 conditions and, finally, in virus neutralization assay using EBOV under BSL4 conditions.

METHODS

Study Area and Sampling
A total of 400 porcine serum samples were collected in 3 different districts of Sierra Leone in 2016 (Supplementary Figure 1). For all samples collected, available information on the pigs’ age, sex, breed, habitat, and housing conditions was recorded. Districts and villages were selected according to the reported presence of EVD cases and the occurrence of pigs in holdings with potential contact to the surrounding wildlife population. No samples were collected in villages without recorded EVD cases. In the Bombali District, 3 villages and several locations in the district's capital Makéni were chosen, whereas 5 villages were selected in Moyamba District. Sampling activities in Port Loko District included the district’s capital, Port Loko, and 4 other villages. Samples were collected according to a Njala University Institutional Review Board protocol (no. IRB00008861/FWA00018924). Sera were heat inactivated for 30 minutes at 60°C before further analysis.

Control Serum Samples
Swine sera from 3 experimentally EBOV-infected animals (pigs P17, P21, and P23) were collected on day 21 and/or 28 postinfection [10] and served as positive controls. Furthermore, to obtain additional control sera, 2 pigs were immunized 4 times each by intramuscular injection in an interval of 3 weeks with 50 µg of insect cell-derived EBOV-like particles (eVLPs) containing viral protein 40 (VP40), glycoprotein (GP), and nucleoprotein (NP) commercially obtained from IBT Bioservices diluted in EMULSIGEN adjuvant (MVP Technologies). Final serum samples were collected 3 weeks after final boost. Ethical approval for animal immunization at the Friedrich-Loeffler-Institut was provided by the competent authority (LALLF 7221.3-2.5-004/10) and the Ethics Committee of the Federal State of Mecklenburg-Western Pomerania, Germany, on the basis of national and European (RL 2010/63/EU) legislation. Serum samples from 150 pigs were collected from different holdings in Germany with specific approval from the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany (LALLF M-V/TSD/7221.3-2.1.014/10, LALLF M-V/TSD/7221.3-2.1-017/13, and LALLF M-V/TSD/7221.3-1.1-022/13) and were used as the negative control panel.

Antigen Expression and Purification
The EBOV NP-coding sequence (Mayinga isolate [16]) was subcloned using the restriction enzymes BamHI and NotI into the pGEX-6P vector (GE Healthcare) resulting in pGEX-6P-NP. The NP sequence was fused in-frame to the glutathione S-transferase (GST), a tag for purification via affinity chromatography after expression in *Escherichia coli*, which was performed as previously described [17].

Indirect Immunoglobulin G Enzyme-Linked Immunosorbent Assay Based on *Escherichia coli*-Derived Ebola Virus Nucleoprotein
Nunc F flat-bottom polystyrene plates were coated with either EBOV NP at a concentration of 200 ng/well (100 µL volume) or with E. coli extract, which served as a mock antigen in control wells to evaluate unspecific binding of the sera. After incubation overnight at 4°C, plates were washed twice (0.05% Tween 20 in 1× phosphate-buffered saline [PBS]; 0.05% PBS Tween 20 [PBST]), and wells were blocked for 2 hours at 37°C using 200 µL/well of blocking buffer (5% goat serum/5% skim milk/0.05% PBST). Plates were then washed as outlined above, and pig serum samples were diluted 1:100 in 2.5% skim milk/0.05% PBST and incubated at 37°C for 1 hour. For serum titration, samples were serially diluted from 1:100 to 1:1600. After the serum incubation, plates were washed 3 times, and a 1:10 000 dilution (in 2.5% skim milk/0.05% PBST) of a goat antipigg antibody conjugated to horseradish peroxidase ([HRP] Dianova; 100 µL/well) was added for 1 hour at 37°C followed by 3 washes. Finally, 100 µL/well of enzymatic substrate (2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); ABTS, Roche Diagnostics) was added. The absorbance at OD405nm was monitored on a Tecan Sunrise absorbance microplate reader until a predetermined OD range of 0.7–0.9 for the positive control sera was reached. The corrected OD405 was calculated by subtracting the nonspecific background OD405 (well coated with mock antigen) from the OD405 of wells coated with recombinant EBOV NP antigen. A cutoff value was established by calculating the mean value of corrected ODs from 150 German porcine sera plus 3 standard deviations, resulting in a cutoff OD405 value of 0.17.

Immunoblot Analysis of Enzyme-Linked Immunosorbent Assay-Reactive Porcine Serum Samples
Sera were tested for reactivity against single formulations of insect cell-derived recombinant EBOV NP [18] and SUDV NP (pAB-bee-FH-SUDV NP [construct obtained from Genesiscript], Gulu isolate) as well as against RESTV NP expressed in 293T cells from a pCAGGS construct [17]. Furthermore, sera were tested against 293T cell-expressed eVLPs (expressing VP40 and GP but lacking the NP). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (3 µg/lane) and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4°C with 5% skim milk in 0.1% Tween 20 in 1× Tris-buffered saline ([TBS] 0.1% TBST) and afterwards incubated with porcine sera (1:20 to 2.5% skim milk/0.01% TBST) for 1 hour at 4°C. After extensive washing with 0.1% TBST, HRP-conjugated goat antiserum antibodies (Dianova) were added to the membrane for 1 hour at room temperature (RT) in a 1:5000 dilution. Three final washes were followed
Neutralization Test Based on Transcription and Replication Competent Virus-Like Particles

The generation of EBOV trVLPs was performed as described previously [19, 20]. Infectivity was determined by serial dilutions of 100 μL trVLP containing supernatant in a 96-well format by infecting 293T cells that were pretransfected with plasmids encoding the T-cell immunoglobulin (Ig) and mucin domain 1 (Tim-1) protein, the EBOV ribonucleoprotein complex proteins [19, 20], and firefly luciferase (for normalization). At 48 hours postinfection, reporter activity was measured. To this end, cells were lysed using 1× lysis buffer (100 μL/well; p.j.k) and incubated for 10 minutes at RT. Then, 40 μL Renilla-Glo Juice (p.j.k) or 40 μL Beetle Juice (p.j.k) in white 96-well plates. Luminescence was measured using a TriStar2 Multimode Reader LB942 (Berthold) with an integration time of 1 second. Renilla luciferase activities in relative light units (RLU) were normalized to control firefly luciferase activities.

For neutralization tests, serum samples were serially diluted in 100 μL modified Eagle’s medium with 5% fetal bovine serum (FBS) MEM5 and subsequently mixed with 300 μL MEM5 containing previously titrated trVLPs. After incubating for 1 hour at 37°C, serum-trVLP-mixtures were added in triplicates to pretransfected 293T cells, as outlined above. After another hour at 37°C, trVLP-antibody mixtures were replaced with 200 μL of fresh MEM with 2% FBS, and cells were further incubated for 48 hours. Reporter activity was then measured, as described above. Neutralizing activity was calculated as follows: (RLU of untreated trVLP control – RLU of serum sample incubated trVLP)/RLU of untreated trVLP control × 100 = percentage inhibition of reporter activity. Serum samples inhibiting reporter activity by 80% or more were considered neutralizing.

Virus Neutralization Assay of Pig Sera

All EBOV infection experiments were performed in the BSL4 laboratory at the Institute of Virology, Philipps University of Marburg, Germany. Virus neutralization assays using EBOV variants Mayinga and Makona were performed as reported previously [21, 22].

RESULTS

The in-house ELISA based on E coli expressed EBOV NP had a specificity of 100% with all 150 German pigs testing negative (data not shown). Because EBOV-positive porcine field samples were missing, the sensitivity was evaluated by analyzing sera from 2 eVLP-immunized pigs and 3 positive porcine sera from experimentally infected animals (provided by the National Centre for Foreign Animal Diseases in Winnipeg, Canada). All of the 5 positive control sera were clearly detected as being positive (ODs ranging from 0.48 to 1.47).

Among the 400 pigs sampled in 2016 in Sierra Leone (Supplementary Figure 1), 194 animals had a reported age of at least 1 year according to the owners. Of those, 57 animals were aged 2 years or older, meaning they were alive during the EVD epidemic in West Africa (2014–2016). Testing of 400 porcine samples from Sierra Leone then revealed 3 serum samples that clearly scored above the cutoff (SL380, SL319, SL241) and 1 serum sample (SL246) that only slightly exceeded the determined threshold with an OD405 of 0.174 (Figure 1A). All 4 pigs were older than 1 year, and their habitat and housing conditions allowed for contact with humans or surrounding wildlife (Supplementary Table 1). The titration of the reactive sera in the ELISA supported specificity of the reaction for 3 sera (SL380, SL319, SL241) and revealed a particularly strong reactivity of sample SL380 against NP up to a dilution of 1:800.
which exceeded the performance of the eVLP control serum (Figure 1B).

In addition, these 4 reactive African sera underwent confirmatory testing by immunoblot analysis. It is interesting to note that 3 African sera clearly detected EBOV NP (Figure 2A), whereas the serum that tested only weakly positive in ELISA (SL246) did not react against EBOV NP in immunoblot analysis, indicating a false-positive ELISA result. Additional sera that tested negative in ELISA were also investigated by immunoblot analysis. None of them specifically bound to the EBOV NP antigen (data not shown), further supporting the ELISA results. To further evaluate the cross-reactivity of the porcine sera, all sera that tested positive in EBOV NP-based assays were analyzed for their reaction against SUDV NP and RESTV NP in immunoblot (Figure 2B, C). Intriguingly, 1 serum from the EBOV animal trial (sample P21) as well as 1 African field serum (SL319) (cross-) reacted with NP from EBOV, RESTV, and SUDV in immunoblot, indicating the presence of antibodies with limited cross-reactivity between the 3 ebolavirus species. In addition, another African field serum (SL380; Figure 2C) reacted with RESTV NP. It is interesting to note that using 293T cell-derived eVLPs containing EBOV VP40 and GP, only the 2 experimental

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Figure 2. Immunoblot analyses of porcine sera from Sierra Leone (SL) and Ebola virus (EBOV) animal experiments (P21, P23) for their reactivity against ebolavirus antigens. Sera were tested against single formulations of recombinant insect cell-derived EBOV nucleoprotein (NP) (A) and Sudan virus (SUDV) NP (B), as well as against 293T cell-expressed Reston virus (RESTV) NP (C) and 293T cell-expressed EBOV-like particles consisting of EBOV viral protein 40 (VP40) and GP (D). SL100 was added as a nonreactive field serum sample. Stars indicate positive reaction in immunoblot analysis.
sera P21 and P23 strongly reacted with GP in immunoblot, in contrast to the field sera (Figure 2D). Also of interest, a less prominent reaction was visible for the experimental sera and some of the African sera against VP40 (Figure 2D).

Finally, to analyze the porcine sera for their neutralizing activity, a trVLP-based serum neutralization test was established that can be handled under BSL1 conditions. After titration of trVLP stocks, a stock-specific trVLP dilution was used that resulted in reporter signals in the infected target cells that were 250 times above background level. Serum from the eVLP-immunized control pig clearly inhibited trVLP entry into the cells. Using a serum dilution of 1:64 (Table 1), reporter activity was still suppressed up to 80% compared with the untreated trVLP control. When compared with the untreated control, inhibition of reporter gene activity of 50% was still observed at a dilution of 1:256 (data not shown). None of the ELISA-reactive African porcine sera inhibited entry of trVLPs and, therefore, resulted in detectable reporter activity comparable to untreated controls. These results fully corresponded to the virus neutralization test results using infectious EBOV under BSL4 conditions (Table 1).

DISCUSSION

Infections with ebolaviruses have caused several EVD outbreaks in humans in Africa in the past 4 decades. Although the virus is zoontic, after an initial spillover event, the main route of transmission during outbreaks is from human to human [1, 23]. Several bat species are widely discussed as serving as a reservoir host [5, 24]. However, pigs have been speculated to play a role in EBOV ecology because RESTV was isolated from Philippine pigs and experimental evidence demonstrated that pigs are susceptible to EBOV infection [8–10]. To further clarify the possible significance of these observations for EBOV ecology, we collected sera from 400 pigs from Sierra Leone in the aftermath of the West African EVD epidemic and tested for the presence of ebolavirus-specific IgG antibodies.

A total of 4 sera from the 400 samples collected in Sierra Leone tested positive in our EBOV NP antigen-based indirect IgG ELISA. Of these, the positive reaction with EBOV NP was confirmed by immunoblot analysis for 3 sera. More importantly, none of the African field sera specifically reacted with EBOV GP in immunoblot or neutralized EBOV trVLPs or live virus, in contrast to the experimental control sera, thus questioning a natural EBOV infection in these African pigs. These results may indicate a previous contact of the pigs with an antigenically related ebolavirus eliciting EBOV NP cross-reactive antibodies. However, some of the porcine sera concurrently reacted with EBOV VP40 in immunoblot, whereas other sera only recognized EBOV NP. Similar findings have been described in another study in which human serum samples were serologically tested for their reactivity with EBOV GP, NP, and VP40. Ninety of 94 human serum samples from EVD survivors were shown to simultaneously react with EBOV GP and NP in the Luminex-based assay, whereas only 87 of 94 samples reacted with all 3 antigens [25]. Furthermore, the level of cross-reactivity against heterologous VP40 was shown to be highly dependent on the ebolavirus species tested [25]. Alternatively, because nothing is known about duration and stability of antibody response against individual viral proteins in pigs, it cannot be excluded that the pigs were infected with a specific ebolavirus, and that antibodies against certain antigens remain detectable for a longer period postinfection than against other antigens. The possibility that the anti-EBOV NP antibody titers may decline shortly after infection was suggested by data presented by Pickering et al [18]. Several other studies have described a serological cross-reactivity against GP or NP antigens between different ebolavirus species when testing sera from bats and humans from different outbreak areas [26, 27]. Further analysis of cross-reactivity of the porcine sera from our study against SUDV NP and RESTV NP by immunoblot analysis revealed that 1 serum from the EBOV animal trial as well as 1 field serum detected the NP of all 3 ebolavirus species, suggesting serological cross-reactivity between ebolavirus species in pigs. Because we found another field serum that only reacted with EBOV and RESTV NP, whereas SUDV NP was not detected, one might speculate that perhaps several antigenically distinct ebolavirus species are circulating in this area. However, cross-reactivity, cross-neutralization, and especially cross-protection of a serum or antibodies against different ebolaviruses

<table>
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<tr>
<th>Serum Sample</th>
<th>Neutralization Titer (SNT trVLPs)</th>
<th>Neutralization Titer (SNT EBOV Mayinga)</th>
<th>Neutralization Titer (SNT EBOV Makona)</th>
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<td>SL241</td>
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<tr>
<td>SL246</td>
<td>&lt;8</td>
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<tr>
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<td>6</td>
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<td>SL380</td>
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<td>64</td>
<td>108</td>
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<tr>
<td>SL260</td>
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Abbreviations: EBOV, Ebola virus; eVLP, EBOV-like particle; GER, Germany; neg, negative; n.t., not tested; pos, positive; SL, Sierra Leone; SNT, serum neutralization test; trVLPs, transcription and replication competent eVLPs.

*Serum from an eVLP-immunized pig as well as sera from pigs from SL and GER were tested for their neutralizing capacity against trVLPs and against EBOV (variant Mayinga and Makona).
vary tremendously, depending on the ebolavirus species studied and the applied test system, and, thus, seroepidemiological studies based on the detection of virus-specific antibodies by ELISA or immunofluorescence should generally be interpreted carefully [27–29]. To precisely discriminate between different ebolavirus species responsible for infection by serological screening, an elaborate diagnostic approach combining several assays and antigens seems necessary.

Furthermore, findings of related filovirus ribonucleic acid and filovirus-specific antibodies in different bat species [30–34] from areas without recorded outbreaks of EVD in humans suggest the circulation of as-yet undiscovered, potentially non-pathogenic filoviruses in nonendemic regions. There is an ongoing debate whether those filoviruses might account for a notable cross-reactive ebolavirus seroprevalence in humans living in these regions [15, 35, 36]. Although RESTV is believed to be a virus of Asian origin, antibodies against RESTV antigens have recently been found in the African straw-colored fruit bat (Eidolon helvum), suggesting the circulation of Reston or Reston-like viruses in a greater geographical range than previously anticipated including Africa [7]. We found at least 2 African pigs with RESTV NP (cross-) reactive antibodies. However, since we observed considerable serological cross-reactivity among the different ebolavirus species tested in our study, it remains to be investigated whether RESTV or Reston-like ebolaviruses circulate in the African pig population. Intriguingly, a 1-year-old African pig (SL380) that tested positive for ebolavirus IgG in our study used to live on a farm with contact to the surrounding bush habitat and potentially to wildlife. Another positive 1-year-old pig (SL319) was reported living free range inside a community, thus enabling contact to both human and wildlife populations. It still remains unclear whether African pigs can play any role as a potential intermediate host transmitting ebolaviruses to humans causing infection.

CONCLUSIONS

In conclusion, we provide the first serological evidence of ebolavirus-specific antibodies in pigs from West Africa, suggesting that African pigs have been in contact with and can be infected by ebolaviruses or ebola-like viruses. However, based on epidemiological evidence, it seems rather unlikely that pigs played an important role in the EBOV ecology in the 2014–2016 EVD outbreak in West Africa. Future comprehensive studies, including animal infection experiments, will be necessary to further understand and define the role of pigs in the ecology of ebolaviruses.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyrighted and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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