Serosurveillance of Viral Pathogens Circulating in West Africa

Aileen E. O'Hearn[1], Matthew A. Voorhees[1], David P. Fetterer[2], Nadia Wauquier[3], Moinya R. Coomber[4], James Bangura[3], Josheph Fair[5], Jean-Paul Gonzalez[3], and Randal J. Schoepp[1]*

[1] Diagnostic Systems Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA

[2] Statistics Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA

[3] Metabiota, Inc., Silver Spring, Maryland, USA

[4] Kenema Government Hospital, Lassa Diagnostic Laboratory, Ministry of Health and Sanitation, Kenema, Sierra Leone

[5]MRI Global, 1330 Piccard Avenue, Rockville, MD, 20850, USA

* Corresponding author:
Randal J. Schoepp
Diagnostic Systems Division
U.S. Army Medical Research Institute of Infectious Diseases
1425 Porter Street
Fort Detrick, Maryland, 21702-5011, USA
301-619-4159
randal.schoepp@us.army.mil

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Abstract

Background

Sub-Saharan Africa is home to a variety of pathogens, however disease surveillance and the healthcare infrastructure necessary for proper management and control are limited. Lassa virus occurs in the West African region, and causes Lassa fever, a severe hemorrhagic fever in humans. At the Kenema Government Hospital in Sierra Leone up to 70% of acute patient samples suspected of Lassa fever test negative for Lassa virus infection, and can be attributed in part to an array of hemorrhagic fever and arthropod-borne viruses. This indicates a substantial amount of disease in the region goes undetected and untreated.

Methods

To further define the nature and extent of viral pathogens burdening the Sierra Leonean population, we developed a multiplexed MAGPIX assay to detect IgG antibodies against Lassa, Ebola , Marburg, Rift Valley fever, and Crimean-Congo hemorrhagic fever viruses as well as pan-assays for flaviviruses and alphaviruses. This assay was used to survey 675 serum samples submitted to the Lassa Diagnostic Laboratory between 2007 and 2014.

Results

In this study population, 50.2% were positive for Lassa virus, 5.2% for Ebola virus, 10.7% for Marburg virus, 1.8% for Rift Valley fever virus, 2.0% for Crimean-Congo hemorrhagic fever virus, 52.9% for flaviviruses and 55.8% for alphaviruses, and evidence of their presence as early as 2007.

Conclusions

These data exemplify the significance of viral hemorrhagic fever differential diagnosis, the importance of disease surveillance, highlight the endemic nature of some of these viral pathogens in Sierra Leone and suggests that unrecognized outbreaks of viral infection have occurred.

List of abbreviations

Lassa virus (LASV)

Lassa fever (LF)

Kenema Government Hospital (KGH)

Ebola virus (EBOV)

Marburg virus (MARV)

Rift Valley fever virus (RVFV)

Yellow fever virus (YFV)

Dengue virus (DENV),

West Nile virus (WNV)

Chikungunya virus (CHIKV)

O'nyong-nyong virus (ONNV)

Immunoglobulin G (IgG)

Japanese encephalitis virus (JEV)

Tick-borne encephalitis virus (TBEV)

Sindbis virus (SINV),

Venezuelan equine encephalitis virus (VEEV)

Western equine encephalitis virus (WEEV)

Eastern equine encephalitis virus (EEEV)

Biological safety level (BSL)

Tissue culture supernatants (TCS)

Enzyme-linked immunosorbent assay (ELISA)

Monoclonal antibodies (MAbs)

Lassa virus glycoprotein complex (GPC)

Lassa virus nucleoprotein (NP)

Ebola virus glycoprotein (GP)

Viral protein 40 (VP40)

Rift Valley fever virus nucleocapsid (NC)

Crimean-Congo hemorrhagic fever virus nucleocapsid (N)

Yellow fever virus envelope protein (E)

Sindbis/Semliki forest glycoprotein E1 (E1)

Room temperature (RT)

Median fluorescence intensity (MFI)

Not applicable (N/A)

Introduction

Sierra Leone experiences a large array of human diseases, but insufficient healthcare infrastructure has left them unrecognized and uncontrolled. Diseases are often only reported as outbreaks and epidemics, despite their consistent presence in the community. A notable exception is Lassa virus (LASV), which is endemic to Sierra Leone and neighboring countries, and causes Lassa fever (LF), a severe viral hemorrhagic fever that can have a case fatality rate as high as 69% (1–4). In eastern Sierra Leone, Kenema Government Hospital (KGH) has a designated LF ward where patients suspected of LASV infection can be isolated and treated. The Lassa Diagnostic Laboratory supports the ward and regional medical facilities and receives approximately 500-700 suspected LF samples annually (1). Of the submitted samples, only 30-40% can be attributed to LASV infection, indicating significant disease resulting from other unidentified pathogens. Studies on acute undiagnosed samples from KGH found evidence of arthropod-borne and hemorrhagic fever virus infections including Ebola virus (EBOV), Marburg virus (MARV), Rift Valley fever virus (RVFV), Yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Chikungunya virus (CHIKV), and O'nyong-nyong virus (ONNV) (5,6), however knowledge on the extent to which the population is burdened by these pathogens is incomplete. To investigate the extent of population exposure to these viruses in Sierra Leone on a population of patients presenting at KGH, we completed a seroprevalence survey with 675 human samples collected at the KGH Lassa Diagnostic Laboratory from suspected LF patients between 2007 and 2014 to detect immunoglobulin G (IgG) antibodies against an array of arthropod-borne and hemorrhagic fever viruses.

We used the magnetic bead-based MAGPIX® system (Luminex, Austin, TX) to detect and identify virus-specific IgG antibodies. In this study, we multiplexed IgG detection assays for antibodies against LASV, EBOV, MARV, RVFV, Crimean Congo Hemorrhagic Fever virus (CCHFV), a pan-flavivirus assay capable of detecting antibodies to an array of flaviviruses including YFV, DENV, WNV, Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV), and a pan-alphavirus assay capable of detecting antibodies to an array of alphaviruses including CHIKV, ONNV, Sindbis virus (SINV), Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV), and Eastern equine encephalitis virus (EEEV).

Materials and Methods

Human samples

The Lassa Diagnostic Laboratory receives blood samples from suspected LF patients and their contacts primarily from Sierra Leone, with occasional samples from Liberia and Guinea. A total of 675 serum samples from suspected LF patients and contacts collected between 2007 and 2014 were tested for IgG antibodies at the Lassa Diagnostic Laboratory and the Diagnostic Systems Division at USAMRIID. Whole blood samples were centrifuged at 200xg, and serum was collected and stored at -80°C until testing. Research on human subjects was conducted in compliance with US Department of Defense, federal and state regulations. All data were gathered and human subjects research was conducted under an institutional review board protocol (no. HP-09-32).

Viral antigen

Viruses used for production of MAGPIX® antigenic materials included LASV Josiah (7,8); EBOV Mayinga (9); MARV Musoke (10); RVFV ZH 501 strain (11); CCHFV IbAr10200 (12); DENV-2 New Guinea C (13,14); WNV NY99 (15); YFV 17D (16); CHIKV B8635. All viruses were propagated at the appropriate biological safety level (BSL), either BSL-3 or BSL-4. Briefly, the viruses were grown in appropriate continuous cell lines until cytopathic effects were observed in 50 to 75% of the cells. Tissue culture supernatants (TCS) were clarified by centrifugation, inactivated by treatment with 0.3% beta-propiolactone, aliquoted, and stored at -70°C. Virus infected TCS was inactivated by gamma-irradiation (3×10^6 rads) and safety tested to ensure inactivation. Optimal dilutions of antigens were determined by checkerboard titrations against virus specific antibodies. Mock TCS antigens used as negative controls were prepared as described above using uninfected cell monolayers.

MAGPIX® Assay Development

Previously, we demonstrated increased sensitivity of the MAGPIX® detection platform over enzyme-linked immunosorbent assays (ELISA) for both LASV and EBOV antigen and antibody detection (17). To develop a multiplexed IgG detection assay to include these and additional viruses, monoclonal antibodies (MAbs) were chosen based on specificity for the target, exclusivity of the additional viruses being tested for, and in the case of the pan-assays, inclusivity of virus family members. Initial antibody selection was based on ELISA checkerboard assessments with the viruses of interest. MAbs with strong affinity for their target were coupled to MAGPIX® magnetic microspheres using the Luminex Antibody Coupling Kit (catalog

#4050016) according to manufacturer's instructions at a concentration of 4µg antibody/1X10^6 beads, optimized for MAGPIX® TCS and IgG detection, and tested for exclusivity against the additional viral targets. Pan-assays were tested and the most family-inclusive antibodies that were exclusive of the additional targets were chosen. MAbs coupled to MAGPIX® microspheres and used in MAGPIX® IgG detection assays were as follows: anti-LASV glycoprotein complex (GPC) L52-85-6-BG12; anti-LASV nucleoprotein (NP) L52-2159-15; anti-EBOV glycoprotein (GP) Z-DA06-AH05; anti-EBOV viral protein 40 (VP40) M-HD6-A10A; anti-MARV VP40 3MI2; anti-RVFV nucleocapsid (NC) R1-P6F6-6-2-2; anti-CCHFV nucleocapsid (N) CCII 12G10-2-2A; anti-YFV envelope (E) YF-211-4E11 (pan-flavivirus capture capable of detecting IgG antibodies to DENV, WNV, YFV, JEV, and TBEV) (18, unpublished data); and anti- SINV/Semliki forest virus glycoprotein E1 (E1) SLK42 (pan-alphavirus capture capable of detecting IgG antibodies to SINV, CHIKV, ONNV, VEEV, WEEV, and EEEV) (19, unpublished data). Individual sets of microspheres are identifiable by unique color signatures detected by the MAGPIX® instrument, permitting the multiplexing of several assays in a single sample well, and assays were combined into one multiplexed assay for IgG detection.

MAGPIX® IgG Detection

Nine distinct tests detecting IgG antibodies against LASV-GP, LASV-NP, EBOV-GP, EBOV-VP40, MARV-VP40, RVFV-NC, CCHFV-N, YFV-E (pan-flavivirus), and SINV-E1 (pan-alphavirus) were combined in a multiplexed IgG detection assay used to test the 675 human sera samples. 2,000 microspheres of each of the nine sets were combined into all wells of 96-well plates. Then, either mock TCS or a mixture of viral infected TCS was added to the wells. The plates

were covered, incubated at room temperature (RT) for 1 hour with shaking at 400-500rpm, and washed three times using a magnetic plate separator. Patient serum was diluted 1:100 and added to wells in triplicate. The plates were covered and incubated at room temperature for 1 hr with shaking. The plates were washed, and anti-human IgG-R-Phycoerythrin (Sigma, St. Louis, MO) was added at a 1:100 dilution. The plates were covered, incubated at room temperature for 30 min, washed, and read on a MAGPIX® instrument. The median fluorescence intensity (MFI) of each bead set in each well was obtained. Throughout the assay, the total volume for each step was 50µl. All dilutions and washes were with 100 µl phosphate-buffered saline with 0.02% Tween.

Statistical Analysis of MAGPIX® Results

Assay results were log transformed prior to analysis. For each sample, the z-score was calculated by the mean difference between the Log transformed sample replicates in viral infected TCS and the Log transformed sample replicates in uninfected TCS, divided by the standard error of the difference. The MFI variance on the Log scale was found to be homogenous within each test, and an appropriate pooled variance estimate was taken across each test in calculating standard errors. Results from multiple tests were collected from a single well, and the data for each test were analyzed separately. This analysis step was carried out with a generalized linear model having identity link and normal distribution, as provided in the SAS® GENMOD procedure (20). Results that had a conservative z-score of at least three standard errors above zero were considered a positive test. Samples testing positive for one or more targets of a single virus was considered positive for the virus; for example, if a sample

tested positive for LASV-NP and negative for LASV-GP, it was considered positive for LASV. Prevalence was calculated using the dichotomization obtained at the three standard errors cut point. Although 675 serum samples were assayed, test results with readings from less than ten beads per well were considered unreliable and therefore excluded from analysis; therefore, the total number of samples analyzed for each test varied.

Results

Prevalence of anti-viral IgG antibodies

A total of 675 serum samples submitted to the Lassa Fever Laboratory in Kenema, Sierra Leone were subjected to serological testing. Samples were tested for IgG antibodies to specific viral targets, and results are presented in Tables 1 and 2. Of the data collected, 50.2% of samples had detectable antibodies to LASV, consistent with previous estimates of 8-52% in the area (21). Antibodies against EBOV were detected in 5.2% (n=35) of the sample population, MARV in 10.7% (n=71), RVFV (n=12, 1.8%) and CCHFV (n=13, 2.0%). Detectable antibodies to one or more flaviviruses was seen in 52.9% of the samples, and 55.8% had detectable antibodies to one or more alphaviruses. Of the seven distinct viruses (including the two pan-assays), the mean number of positive tests was 1.8, with 26.2% of individuals testing positive for 3 or more distinct viruses. A more accurate number is likely higher, considering the pan-alphavirus and pan-flavivirus tests detect multiple exposures.

Longitudinal assessment of prevalence

A longitudinal assessment was carried out to investigate chronological trends in positive IgG antibody rates (Table 3). Surprisingly, MARV antibodies were detected in 23% of samples tested from 2008, suggesting a possible unrecognized outbreak of the virus in the area. Of additional note is the large increase in RVFV in 2014, and the steady decline of alphavirus positive rates, which may represent the slow recovery from the ONNV outbreak that occurred in the region in 2003 (22). It should be noted that year 2009 had only one representative sample and was therefore not included in the table below.

Discussion

Knowledge of the diseases circulating in a region is paramount for proper diagnosis, care and treatment of patients, and ultimately a reduction of overall disease burden. Sierra Leone and the surrounding areas suffer from numerous viral diseases, but surveillance and diagnostic capabilities fall short of the need. Moreover, as demonstrated by the recent EBOV outbreak in West Africa, differential diagnosis among VHFs and knowledge of their endemicity is of great importance for timely and efficient management of patients and outbreak prevention.Here we applied a multiplexed serological assay to screen a panel of 675 serum samples from Sierra Leone to identify the extent and nature of viral burden in the region.

Among suspected LF patients tested for exposure to LASV and additional pathogens, we report the seroprevalence of LASV to be 50.2%; this shows little change from estimations in Sierra Leone before the civil war, which ranged from 8-52% throughout the country and peaking in the Eastern region, where KGH is located (21). EBOV and MARV were found at 5.2% and 10.7%, respectively. A recent study estimated seroprevalence of filoviruses to be 22% in

the area, and an IgM survey from the same hospital reported a 9% acute EBOV infection rate between 2006 and 2008 (5,6). Considering the estimated fatality rates of EBOV and that we are observing latent immunity only present in survivors, the 5.2% prevalence seen here correlates appropriately with the 9% observed acute cases during the time period of sample collection. Exposures EBOV are detected in the population as early as 2008 and display a consistent presence throughout the time period tested, suggesting a reservoir that has been maintaining EBOV in the environment. We found the overall prevalence of MARV to be 10.7% and present as early as 2007; although there are no records of MARV outbreaks or disease in this region, it was found retrospectively in 3.6% of acute samples from KGH dating from 2006-2008, suggesting an increase in exposures occurred in the last seven years. There is a notable jump in MARV to 23% seroprevalence in 2008, suggesting a possible unrecognized outbreak of the MARV in the area or exposure to the antigen.

RVFV is a bunyavirus known to circulate throughout sub-Saharan Africa, mainly among livestock and *Aedes* species mosquitos, with sporadic outbreaks of human disease. While the climate and epizootic factors are well studied in East and South Africa, it has been postulated that there are other factors supporting endemic sustainability in West African regions (23–25). Here we report a seroprevalence of 1.8%, and observed a notable increase in prevalence from 1-3% in 2007-2013 to 11% in 2014, indicating a substantial and recent increase in its circulation. Another bunyavirus, CCHFV, is known to occur throughout Africa, Asia and Europe in animals and ticks. Human infection, albeit rare, is severe and usually associated with livestock contact (26–29). Here we detect low numbers of CCHFV exposure (n=13, 2.0%) , even though it was not detected in the area in recent studies (5,6). The relatively low but consistent prevalence of

antibodies to RVFV and CCHFV may suggest the presence of reservoirs or continual reintroduction to the region resulting in low but consistent levels of human infection.

Flavivirus and alphavirus serology is difficult to interpret since there is significant cross-reactivity among viruses within their respective families. Generally, antibodies to a specific virus must be distinguished by plaque reduction neutralization tests and/or molecular testing. It is known that multiple alphaviruses and flaviviruses circulate in this region, some described and some yet undiscovered, but our interests were in the prevalence of the overall disease attributed to each group. Therefore given the number of samples tested here and the desire for broad and complete surveillance, we developed two pan-assays intended for the widest possible coverage of flavivirus and alphavirus species. Antibody prevalence rates were high for viruses of both families; combined prevalence of flavivirus and alphavirus antibodies were 52.9% and 55.8%, respectively. There are limited data on the seroprevalence to flaviviruses in Sierra Leone. A survey by Boisen et al. (n=77) from the same hospital revealed 45% seroprevalence to DENV and 54% to WNV (6). Studies in the neighboring countries of Guinea, Nigeria, and Cameroon report a range of seroprevalence to flaviviruses including YFV (27-43%), DENV-2 (12-45%) and WNV (7-49%) (30–32), each of which are able to be detected in the pan-assay utilized here and likely represent significant portions. Also, it is possible that YFV vaccination may have impacted the prevalence of flaviviruses recorded here; however, distribution of the vaccine is notably irregular and a measure of its impact is likely unreliable. Similar to the flaviviruses, there is limited information on the prevalence of alphavirus antibodies in Sierra Leone. Boisen et al. reported in the same survey of 77 KGH serum samples a prevalence to CHIKV of 27% (6). The 55.8% prevalence of alphavirus antibodies is similar to

287 estimates of CHIKV and ONNV in nearby Cameroon where approximately 47% of healthy adults
288 tested positive for CHIKV and/or ONNV (with noted overlap) (32). Our longitudinal data
289 revealed a high prevalence of alphaviruses prior to 2010 (>60%), which may be in part due to
290 ONNV and CHIKV outbreaks known to occur in Guinea in 2003 (22) and 2006 (33). Additionally,
291 a spike in 2012 and a subsequent decrease in the following years correlates with a reported
292 CHIKV outbreak in Sierra Leone in 2012, identified in a hospital only 60 kilometers from KGH
293 (34). The high rates of both flaviviruses and alphaviruses seen here, combined with identified
294 recurring outbreaks of CHIKV and ONNV, highlight the range of endemic viruses and their
295 significant impact on the limited medical infrastructure.

296 Multiplexed and bead-based platforms such as the MAGPIX utilized here can provide
297 valuable information by evaluating disease burden and risk in regions where this information is
298 not monitored. As demonstrated here, assays can be customized with relative ease to survey
299 for many different targets. With further evaluation, they can be suitable for diagnostic
300 confirmation using detection of IgM and antigen, as demonstrated for LASV and EBOV
301 detection (17).

302 Retrospective studies have limitations by their very nature. In this study the samples
303 tested had a bias for subjects that 1) were willing to seek help from the hospital, and 2) had at
304 some point presented with symptoms resembling LF. An individual's presentation of LF may
305 indicate they are more likely to be exposed to pathogens via factors in their lifestyle,
306 geographic location, or workplace environment. Overall, our results indicate that in addition to
307 LASV, there is a significant presence of filoviruses, bunyaviruses, flaviviruses and alphaviruses

actively circulating in the Sierra Leone and the surrounding regions, and evidence of such as early as 2007. Additionally, 26.2% of this study population were positive for exposure to at least three of the viruses tested for, indicating a severe public health burden. The prevailing nature of some pathogens over the entire seven-year timespan tested here suggests possible longstanding reservoirs and endemicity. Further, we found indications of possible unrecognized outbreaks of infection, or subclinical exposure. Increased surveillance methods as described here utilized in Sierra Leone and elsewhere will be a useful tool to improve the diagnosis and control of these diseases.

Competing interests

The authors declare no competing interests.

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329 **Authors' contributions**

330 AEO contributed to experimental design, experimental execution, data collection, data analysis,
331 and drafting of manuscript. MAV contributed to experimental design, experimental execution,
332 data collection, and data analysis. DF contributed extensive data analysis and critical review of
333 manuscript. NW and MRC provided laboratory and experimental assistance at KGH. JB, JF, JPG,
334 and RG provided necessary aid in Sierra Leone and access to samples. RJS contributed to
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344 Research on human subjects was conducted in compliance with DoD, Federal, and State
345 statutes and regulations relating to the protection of human subjects, and adheres to principles
346 identified in the Belmont Report (1979). All data and human subjects research were gathered
347 and conducted for this publication under an IRB approved protocol, number HP-09-32.

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Analyte	Positive/total tested (%)
LASV	328/654 (50.2%)
EBOV	35/672 (5.2%)
MARV	71/663 (10.7%)
RVFV	12/667 (1.8%)
CCHFV	13/641 (2.0%)
Pan-alphavirus	330/624 (52.9%)
Pan-flavivirus	373/668 (55.8%)

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Table 1. Seroprevalence of each target virus among samples obtained from suspected LF patients. Number of samples testing positive, total number tested, and percent tested positive by MAGPIX.

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Number of positive tests per sample	Frequency (n)	Percent (%) of total samples
0	109	16.15
1	138	20.44
2	174	25.78
3	137	20.30
4	29	4.30
5	6	0.89
6	4	0.59
7	1	0.15
N/A	77	11.41

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Table 2. Distribution of number of positive tests identified per sample, out of seven distinct tests (LASV, EBOV, MARV, RVFV, CCHFV, Pan-alphavirus, and Pan-flavivirus). Samples that did not have valid results for all 7 distinct tests are listed as not applicable (N/A).

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	Seroprevalence rate (% of total each year)							
Year	**Total samples**	**LASV**	**EBOV**	**MARV**	**RVFV**	**CCHFV**	**Pan-flavivirus**	**Pan-alphavirus**
2007	51	41	0	8	0	0	49	61
2008	151	57	3	23	1	1	38	68
2010	195	51	7	7	1	2	67	65
2011	153	36	5	6	3	3	39	32
2012	66	67	10	13	1	1	64	63
2013	41	61	2	0	0	2	73	49
2014	19	37	0	0	11	5	47	26

442 Table 3. Observed seropositive rates are reported in percent of total samples from that year for each pathogen. There was only one representative sample from the year 2009, therefore statistics for that year are not included in the chart.

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