Comparative evaluation of the prototype Cepheid GeneXpert® Ebola Assay diagnostic performance

Petrus Jansen van Vuren[1,2], Antoinette Grobbelaar[1], Nadia Storm[1],Ousman Conteh[3],Kelfala Konneh[3],Abdul Kamara[3], Ian Sanne[4,5], Janusz T. Paweska[1,2,6]*

[1] Centre for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases, National Health Laboratory Service, Sandringham, South Africa;

[2]Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Science, University of Pretoria, South Africa;

[3]Ministry of Health and Sanitation, Freetown, Sierra Leone;

[4] Clinical HIV Research Unit, Wits Health Consortium, Johannesburg, South Africa;

[5] Right-to-Care, Johannesburg, South Africa;

[6]Faculty of Medical Sciences, University of the Witwatersrand, Johannesburg, South Africa;

* Corresponding author:

Janusz T. Paweska, Prof. dr. hab.

Private Bag X4

Sandringham, Johannesburg, 2131

South Africa

januszp@nicd.ac.za

ABSTRACT

The Ebola virus disease (EVD) outbreak in West Africa has highlighted an urgent need for point-of-care assays (POC) for the diagnosis of this devastating disease in resource-limited African countries. Diagnostic performance characteristics of a prototype Cepheid GeneXpert® Ebola POC to detect Ebola virus (EBOV) in stored serum and plasma samples collected from suspected EVD cases in Sierra Leone, 2014 and 2015, was evaluated. The GeneXpert® Ebola POC is a self-contained single cartridge automated system targeting the glycoprotein (GP) and nucleoprotein (NP) genes of EBOV, and yielding results within 90 minutes. Results from 281 patient samples were compared to results of a TaqMan real-time RT-PCR targeting the polymerase gene, performed on two real-time PCR machines. Agreement between the three platforms was 100% at CT values up to 34.99, but discordant results were noted between CT 35 and 45.The diagnostic sensitivity of the three platforms was 100% in 91 patient samples confirmed to be infectious by virus isolation. All three molecular platforms detected viral EBOV RNA in additional samples not containing viable EBOV. Analytical sensitivity of the GeneXpert® Ebola POC for the detection of NP was higher, and comparable to that of polymerase gene detection, than for the detection of GP when using titrated laboratory stock of EBOV. There was no detectable cross-reactivity with other haemorrhagic fever- and arboviruses. The GeneXpert® Ebola POC offers an easy to operate and sensitive diagnostic tool that could be used for rapid screening of suspected EVD cases in treatment or holding centres during EVD outbreaks.

Keywords: Ebola virus disease; point-of-care diagnosis; real-time PCR; diagnostic accuracy, filovirus

INTRODUCTION

The unprecedented scale of Ebola virus disease (EVD) outbreaks in West Africa in 2013-2015 caused by Ebola virus (EBOV), represents a dramatic expansion of case numbers and introduction of this highly lethal disease into new geographic areas (1-2). As of October 07, 2015 the World Health Organization reported a total of 28,421 EVD cases (confirmed, probable and suspected) of which 11,297 (39.7%) were fatal, including 881 confirmed cases among health care workers of which 513 (58.2%) died (3). The diagnostic burden of the largest EVD outbreak in the recorded history of the disease has been mostly borne by mobile laboratories, deployed throughout the affected countries by international agencies and institutes. Delays in diagnosis of suspected EVD cases due to sample transport from remote areas to these laboratories have put additional pressure on outbreak control efforts. The use of a wide range of assays, often not clinically validated, has complicated the interpretation and consolidation of results from different laboratories. Rapid and accurate diagnostic results have a great impact on the management of suspected cases and tracing of contacts. The extent to which qualitative cycle-threshold (Ct) values from real-time reverse transcription polymerase chain reaction (RT-PCR) assays correlate with the infectious state of patients is not well understood. Patients are often kept in isolation until three consecutive blood samples, collected days apart, are found negative by RT-PCR. High Ct value results are often recorded in blood of recovering patients for several days after clinical recovery (NICD unpublished data). It is unclear whether these high Ct values infer that the patient still represents a risk for spreading infection.

The 2013-2015 West African EVD outbreak is caused by Ebola virus (species *Zaire ebolavirus*), one of five species in the *Ebolavirus* genus which is known to cause high fatality rate in infected humans (60-90%) (4). The EBOV genome is negative sense, single-strand RNA consisting of 18959 nucleotides, encoding seven structural and one non-structural

protein. After 2-21 days of incubation, the disease presents initially with flu-like symptoms such as fever, malaise and myalgia followed by vomiting, diarrhoea, abdominal pain, oedema, neurological signs and haemorrhagic manifestations such as rash, petechiae and bleeding from puncture sites (4).The non-specific clinical presentation of viral haemorrhagic fevers (VHFs) and their high risk for nosocomial spread highlight the importance of accurate and rapid laboratory diagnosis. Diagnosis of infection by a filovirus can be achieved by detection of antigen, virus nucleic acid, isolation of virus or detection of a virus specific antibody response. Antigen can be detected in serum and other body fluid samples by antigen detection ELISA (5) and indirect immunoelectron microscopy (6), and in skin biopsies by immunohistochemistry (7).Detection of immunoglobulin (Ig) M and G specific to filoviruses can indicate a recent or past infection (8). Traditionally filoviruses have been isolated successfully *in vitro* in African green monkey cell cultures (Vero) (9) and suckling mice (10). In recent years filovirus diagnostics have relied mostly on real-time RT-PCR assays using fluorogenic probes (11-19).Two prototype point-of-care assays that detect viral antigen by using lateral flow technology were recently evaluated using clinical specimens from Sierra Leonean suspected EVD cases (20,21).

In this study we evaluated the diagnostic performance of the prototype GeneXpert® (Cepheid, Sunnyvale, California, United States) Ebola assay in serum and plasma samples from suspected EVD cases in Sierra Leone. Results of this assay were directly compared to real-time RT-PCR targeting the polymerase (L) gene, run on two field deployable real-time PCR platforms, SmartCycler® (Cepheid, Sunnyvale, California, United States) and LightCycler® Nano (Roche, Basel, Switzerland).Sensitivity of the three molecular diagnostic platforms was compared to virus isolation in Vero E6 cell culture.

MATERIALS AND METHODS

Analytical sensitivity. To determine and compare the analytical sensitivity of the GeneXpert® Ebola assay to the polymerase gene based TaqMan RT-PCR run on two real-time platforms, a log dilution series of stock Ebola virus (SPU220/96, passage 4 Vero, 1 x $10^{5.0}$ $TCID_{50}$/mL) was prepared in culture medium (EMEM) and tested in quadruplicate.

Analytical specificity. To evaluate the cross-reactivity of the GeneXpert® Ebola assay with selected haemorrhagic fever and arthropod borne (arbo) viruses, stocks of the following viruses were tested: Sudan and Marburg viruses (*Filoviridae*); Lassa and Lujo viruses (*Arenaviridae*); Rift Valley fever and Crimean-Congo haemorrhagic fever viruses (*Bunyaviridae*); West Nile, Yellow fever and Dengue type 1-4 viruses (*Flaviviridae*); Chikungunya and Sindbis viruses (*Alphaviridae*). More detailed individual isolate information can be found in Table 1.

Diagnostic sensitivity. Diagnostic sensitivity was evaluated using 281 blood specimens from suspected EVD cases submitted from August 2014 through March 2015 to the field Ebola Molecular Laboratory of the Centre for Emerging and Zoonotic Diseases of National Institute for Communicable Diseases (NICD) in Freetown, Sierra Leone. This field laboratory was established as a part of the WHO Global Outbreak Alert and Response Network international outbreak response. Serum was separated from clotted blood and plasma from EDTA and stored at -70°C before shipment on dry-ice to the NICD biosafety level 4 facility (BSL-4) in Johannesburg, South Africa for further analysis and long term storage.

Table 1.Cross-reactivity of the GeneXpert Ebola assay with other haemorrhagic fever viruses and arboviruses.

Family	**Virus**	**Isolate**	**Virus concentration per mL**	**GeneXpert result (GP and NP target)**
Filoviridae	Sudan virus	276/00/6 (passage 2 Vero)	2.2 x $10^{8.0}$ RNA copies	negative
	Marburg virus	Watsa/DRC 148/99/1 (passage 2 Vero)	1 x $10^{5.75}$ $TCID_{50}$	negative
Arenaviridae	Lassa virus	Luga L319 (passage 5 Vero)	1 x $10^{6.7}$ FFU	negative
	Lujo virus	GM serum (passage 5 Vero)	1 x 10^{8} FFU	negative
Bunyaviridae	Rift Valley fever virus	1981 V20368 (passage 1 BHK)	1 x $10^{6.75}$ $TCID_{50}$	negative
	Crimean-Congo hemorrhagic fever virus	SPU4/81 (passage 21 Vero)	1 x $10^{7.6}$ $TCID_{50}$	negative
Flaviviridae	West Nile virus	SPU 116/89 (passage 5 Vero)	1 x $10^{8.25}$ $TCID_{50}$	negative
	Yellow fever virus	A9/86	1 x $10^{5.25}$ $TCID_{50}$	negative

		(passage 2 Vero)		
	Dengue virus serotype 1	Prototype TVP 2172 3/22/89 (passage 2 Vero)	5.4 x $10^{6.0}$ RNA copies	negative
	Dengue virus serotype 2	NGC TVP 10863 7/2/2011 (passage 2 Vero)	1.6 x $10^{6.0}$ RNA copies	negative
	Dengue virus serotype 3	H87 TVP 17541 8/10/2012 (passage 2 Vero)	4.7 x $10^{6.0}$ RNA copies	negative
	Dengue virus serotype 4	SA216/15 (passage 1 Vero)	RNA copies unknown (Ct 17.22)	negative
Alphaviridae	Chikungunya virus	H817 (passage 11 C6-36)	1 x $10^{7.5}$ $TCID_{50}$	negative
	Sindbis virus	AR86 (passage 10 C6-36)	1 x $10^{8.5}$ $TCID_{50}$	negative

$TCID_{50}$ = tissue culture infectious dose 50; FFU = fluorescence focus units;

Extraction of viral RNA. RNA was extracted from clinical and laboratory generated samples (input 140 µL) using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) per the manufacturer's instruction and as previously described (22). Final elution volume was 60 µL.

Polymerase (L) gene TaqMan real-time RT-PCR. The assay was performed as previously described (14, 22), using the primers and probe targeting the Ebola virus L-gene and 5 µL RNA as template. An *in-vitro* transcribed RNA copy of the L-gene was used as a positive control at a known copy number. The RNA standard was prepared as previously described (23) using L -gene specific primers (14). All runs included two negative controls as per standard PCR practice: a no-template control and an extraction negative control. Two field-deployable real-time PCR platforms were used: SmartCycler® (Cepheid) using propriety single reaction Smart Tubes and LightCycler® Nano (Roche) using 8-well strip tubes. The SmartCycler is operated with Cepheid Smart Cycler Version 2.0d software, and the LightCycler Nano with software version 1.0.7. Analysis on both platforms' software was run with default analysis settings. Cycles were as follows: reverse transcription (50°C for 30 min), denaturation (95°C for 15 min) and amplification/detection (45 cycles of 95°C for 15 sec, 52°C for 25 seconds plus acquisition, 72°C for 20 sec). Any fluorescence detected above the threshold before 45 cycles is regarded as positive by the software and assigned a Ct value. Both these platforms were deployed in the NICD-EMLU in Sierra Leone from August 2014 to run the polymerase gene TaqMan real-time RT-PCR. The SmartCycler results were used to determine RNA copy numbers in patient samples.

GeneXpert® Ebola assay. The assay was performed on the GeneXpert® IV system and Cepheid GeneXpert® Dx software package using disposable prototype GeneXpert® Ebola cartridges. The assay integrates sample purification, nucleic acid amplification and detection

of target sequence in a single automated process. In addition to targeting EBOV nucleoprotein (NP) and glycoprotein (GP) genes, the assay includes a sample adequacy control (SAC – human housekeeping gene hydroxymethylbilane synthase HMBS) and internal control (IC) to ensure adequate addition of sample and to control for PCR inhibitors. A volume of 100 µL serum or plasma (or laboratory generated samples) was added to the lysis reagent bottle (containing guanidinium thiocyanate) in the BSL-4 laboratory. Outside biocontainment a volume of 1 mL of the sample/lysis mix was added directly to the GeneXpert® Ebola cartridge sample well and processed on the GeneXpert® IV system. The cycle threshold (Ct) values for NP and GP were obtained through the GeneXpert® Dx software package, along with a decision on the validity of a specific test based on the two internal controls (SAC and IC). Any fluorescence detected above the threshold with either target (NP or GP) before 45 cycles is regarded as positive by the software and assigned a Ct value.

Virus isolation. Serum or plasma samples that tested positive either by one or all of the abovementioned molecular assays were subjected to virus isolation. Samples were diluted 1:5 in tissue culture medium (EMEM) prior to inoculation. Vero E6 cells at 80-90% confluency in 25cm^2 flasks were overlaid with 1 mL diluted sample and incubated for 1 hr at 37°C. After removal of the inoculum, fresh EMEM containing antibiotics (Penicillin/Streptomycin/AmphotericinB) was added (10 mL) and the flasks incubated at 37°C for 14 days or until cytopathic effects (CPE) were observed. After incubation (or at early signs of CPE), flasks were frozen at -70°C and subsequently thawed at room temperature. Presence or absence of replicating virus in the culture supernatants was confirmed by real-time RT-PCR. Cultures in which the virus did not replicate in the first

passage were subjected to a second passage by inoculating 1 mL of undiluted supernatant from passage one, followed by incubation and testing, as described above.

Statistics. Percentage agreement was calculated between different assays and virus isolation. Diagnostic accuracy parameters for the assays at 95% confidence interval (CI) were calculated using MedCalc version 15.8 (www.medcalc.org). The following estimates were calculated: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Basic calculations of means and standard deviations were done in Microsoft Excel 2007.Cut-off values for determining the Ct value at which a patient sample is likely to yield an isolate on VeroE6 cells, at the 95 % accuracy level, using different gene targets or assay platforms, were optimized using the two-graph receiver operating characteristics (TG-ROC) analysis (24-26).

Ethics Statement. Approval to conduct this study was obtained the Office of Sierra Leone Ethics and Scientific Review Committee (version 24/03/2015) and from the Human Research Ethics Committee of the University of the Witwatersrand, South Africa (clearance certificate number M150157). Clearance for export of samples from Sierra Leone to South Africa was granted under export permit numbers PBSL/061/02/2015 and PBSL/063/02/2015 by the Pharmacy Board of Sierra Leone. Xpert® Ebola assay (Cepheid) has WHO authorization for emergency use.

RESULTS

Analytical sensitivity and specificity. Ten-fold serial dilutions of live EBOV in culture medium from 10,000 to 0.01 $TCID_{50}$/mL were tested in quadruplicate using the GeneXpert Ebola assay and the TaqMan RT-PCR targeting the virus polymerase (L) gene on the

Table 2. Analytical sensitivity of GeneXpertEbola assay vs. L-gene qRT-PCR on SmartCycler and LightCycler Nano using serial dilutions of Ebola virus at known titres in tissue culture medium.

Stock virus titration			**Ebola GeneXpert assay**				**SmartCycler**		**LightCycler Nano**	
Ebola virus 220/96 #4 Vero[a] $TCID_{50}$/mL	**RNA copies per mL[b]**	**Ratio RNA copies to $TCID_{50}$**	**GP Replicates[c] Positive**	**Ct (±StDev)[d]**	**NP Replicates Positive**	**Ct (±StDev)**	**Replicates Positive**	**Ct (±StDev)**	**Replicates Positive**	**Ct (±StDev)**
10000	7020300	702	4/4	29.03 ± 0.22	4/4	24.4 ± 0.22	4/4	25.3 ± 0.19	4/4	21.9 ± 0.13
1000	624120	624	4/4	32.68 ± 0.21	4/4	28.25 ± 0.1	4/4	28.9 ± 0.12	4/4	25.5 ± 0.55
100	31773	318	4/4	36.03 ± 0.37	4/4	31.68 ± 0.21	4/4	33.4 ± 0.36	4/4	28.9 ± 0.33
10	3660.3	366	4/4	39.83 ± 0.95	4/4	35.33 ± 0.1	4/4	36.7 ± 0.51	4/4	32.7 ± 0.09
1	163.52	163	1/4	41.50	4/4	38.2 ± 0.08	4/4	42.1 ± 1.75	4/4	35.2 ± 0.26
0.1	73.92	739	0/4	-	1/4	41.3	1/4	41.1	2/4	41.2; 39.5
0.01	0	n/a	0/4	-	0/4	-	0/4		0/4	

[a] Ebola virus isolate 220/96, passage four on Vero cells, at a known titre of 1 x 10^5 $TCID_{50}$/mL was used to prepare tenfold serial dilutions in tissue culture medium.

[b] RNA copies calculated using the data obtained on the SmartCycler.

[c] Each dilution of the stock virus was tested in quadruplicate. Number positive out of number tested is shown.

[d] The average Ct value is indicated at each dilution. Standard deviation is shown where 4 replicates yielded a Ct value.

SmartCycler and LightCycler Nano platforms (Table 2). The L-gene based RT-PCR on both platforms detected virus RNA in all four replicates at 1.0 $TCID_{50}$/mL. The Ct value obtained with the LightCycler Nano was consistently lower than the value obtained with the SmartCycler at the same dilution (smallest and largest difference 3.4 and 6.9 Ct values respectively). At 0.1 $TCID_{50}$/mL the LightCycler Nano yielded fluorescence in two of the four replicates compared to one replicate with the SmartCycler. Although the GeneXpert also detected the NP target in all four replicates at 1.0 $TCID_{50}$/mL, the GP target could only be detected in all replicates down to 10 $TCID_{50}$/mL. An additional single replicate yielded detectable NP target at 0.1 $TCID_{50}$/mL and GP target at 1.0 $TCID_{50}$/mL respectively. No cross-reaction could be detected using the GeneXpert Ebola assay with other haemorrhagic fever viruses and arboviruses tested (Table 1).

Diagnostic accuracy. A direct comparison was done between the GeneXpert Ebola assay and the L-gene TaqMan assay on two platforms, using two different Ct cut-off values. Using the L-gene assay run on the SmartCycler platform as a comparator, percentage of agreements were calculated (Table 3). A total of 122 samples were regarded as positive and 159 as negative when using a Ct cut-off value of 45, compared to 112 positive and 169 negative when using a Ct of 40. The agreement between the assays was the highest when using a Ct of 45 as cut-off. When analysing the data separately for the two targets in the GeneXpert assay, the agreement of the GP target was lower than with NP regardless of cut-off value used. The agreement was highest between the L-gene TaqMan assay run on the two different platforms at a 45 Ct cut-off, but not at 40 Ct. Using the lower cut-off of 40 Ct decreased the sensitivity (from 99.18% to 97.32%) and increased specificity (from 97.48% to 98.22%) of the GeneXpert (one or both targets) compared to analysis with a 45 Ct cut-off, but had the

Table 3. Comparison of the GeneXpertEbola assay and L-gene qRT-PCR on LightCycler Nano and SmartCycler using clinical samples collected from suspected EVD cases in Freetown, Sierra Leone, 2014-2105.

	GeneXpertEbola assay						LightCycler Nano L-gene qRT-PCR	
	GP target		NP target		One or both targets (GP & NP)			
	+	-	+	-	+	-	+	-
SmartCycler L-gene qRT-PCR Positive (n = 122)[a] (n = 112)[b]	107[a] 97[b]	15[a,b]	120[a] 109[b]	2[a] 3[b]	121[a] 109[b]	1[a] 3[b]	120[a] 111[b]	2[a] 1[b]
SmartCycler L-gene qRT-PCR Negative (n = 159)[a] (n = 169)[b]	2[a] 0[b]	157[a] 169[b]	3[a,b]	156[a] 166[b]	4[a] 3[b]	155[a] 166[b]	1[a] 10[b]	158[a] 159[b]
Agreement	93.95%[a] 94.66%[b]		98.22%[a] 97.86%[b]		98.22%[a] 97.86%[b]		98.93%[a] 96.09%[b]	

[a] Ct cut-off value of ≤ 45 used

[b] Ct cut-off value of ≤ 40 used

Table 4. Agreement between the GeneXpertEbola assay, and L-gene qRT-PCR on LightCycler Nano and SmartCycler at different Ct value ranges, using clinical samples collected from suspected EVD cases in Freetown, Sierra Leone, 2014-2015.

SmartCycler L-gene qRT-PCR Ct value range	GeneXpertEbola assay						LightCycler Nano L-gene qRT-PCR	
	GP target		NP target		One or both targets (GP & NP)			
	+	-	+	-	+	-	+	-
Ct< 30 (n = 81)	81	0	81	0	81	0	81	0
	100% [a]		100%		100%		100%	
Ct 30 – 34.99 (n = 15)	15	0	15	0	15	0	15	0
	100%		100%		100%		100%	
Ct 35 – 39.99 (n = 16)	9	7	15	1	15	1	15	1
	56.25%		93.75%		93.75%		93.75%	
Ct 40 – 45 (n = 10)	2	8	9	1	10	0	9	1
	20.0%		90.0%		100%		90.0%	
No fluorescence signal above the threshold (n = 159)	2	157	3	156	4	155	1	158
	98.74%		98.11%		97.48%		99.37%	

[a] Percentage agreement between applicable assay and SmartCycler L-gene qRT-PCR using a cut-off of Ct 45.

inverse effect when analysing the LightCycler Nano platform results (sensitivity from 98.36% to 99.11% and specificity from 99.37% to 94.08%).

Samples were further categorized according to range of Ct values obtained by L-gene TaqMan assay on the SmartCycler. At Ct values ≤ 34.99, there was a 100% agreement between all assays and platforms (Table 4). At Ct values between 35 and 39.99, the agreement decreased slightly (93.75% for all assays), but was much lower when analysing specifically the GP target of the GeneXpert Ebola assay (56.25%). Between Ct 40 and 45 the agreement was 90% between L-gene TaqMan run on SmartCycler and LightCycler, 100% between GeneXpert Ebola assay and L-gene SmartCycler but only 20% and 90% when analysing the two targets GP and NP respectively.

Of a total of 125 serum samples subjected to virus isolation, Ebola virus could be recovered from only 91 samples after a maximum of two passages on VeroE6. The agreement of RT-PCR results on all three platforms to virus isolation as standard reference was analysed (Table 5). Agreement in samples from which EBOV could be isolated was 100% with all assays when using Ct 45 as cut-off. At Ct cut-off of 40 only the agreement with the GP target in the GeneXpert assay decreased to 98.9%, while the others remained 100% in samples from which EBOV was isolated. Agreement in virus isolation negative samples was poor and varied between 2.9% and 82.35% depending on assay and cut-off used.

Infectivity versus Ct values. The Ct values obtained by the different assays were compared to virus isolation results. Two samples yielded clearly outlying results. Virus could not be isolated from one sample (date of collection post onset unknown) yielding the following Ct values on the different assays: SmartCycler 26.92; LightCycler Nano 25.64; GeneXpert GP 28.5 and NP 25.1. Another sample (sample collected on day 12 post-onset) yielded live virus but the following Ct values: SmartCycler 38.62; LightCycler Nano 34.76; GeneXpert GP

Table 5. Agreement between qRT-PCR based assays and virus isolation from suspected EVD cases in Freetown, Sierra Leone, 2014-2015.

	GeneXpert Ebola assay						LightCycler Nano L-gene qRT-PCR		SmartCycler L-gene qRT-PCR	
	GP target		NP target		One or both targets (GP & NP)					
	+	-	+	-	+	-	+	-	+	-
Virus isolation positive (n = 91)	91[a] Ct range (22.2-40.5) 90[b] Ct range (22.2-36.8)	0[a] 1[b] CT 40.5	91[a,b] Ct range (15.8-38.2)	0[a,b]	91[a,b] Ct range (15.8-40.5)	0[a,b]	91[a,b] Ct range (13.2-34.76)	0[a,b]	91[a,b] Ct range (14.77-38.62)	0[a,b]
Virus isolation negative (n = 34)	17[a] Ct range (28.5-44.6) 6[b] Ct range (28.5-38.8)	17[a] Ct > 45 28[b] Ct > 40	31[a] Ct range (25.1-43.8) 20[b] Ct range (25.1-39.8)	3[a] Ct > 45 14[b] Ct > 40	33[a] Ct range (25.1-44.6) 20[b] Ct range (25.1-39.8)	1[a] Ct > 45 14[b] Ct > 40	19[a,b] Ct range (25.6-39.8)	5[a,b] Ct > 40	30[a] Ct range (26.9-42.9) 20[b] Ct range (26.9-39.96)	4[a] Ct > 45 14[b] Ct > 40

Agreement Virus isolation positive	100%[a] 98.9%[b]		100%[a,b]		100%[a,b]		100%[a,b]		100%[a,b]	
Agreement Virus isolation negative	50.0%[a] 82.35[b]		8.8%[a] 41.2%[b]		2.9%[a] 41.2%[b]		14.3%[a,b]		11.8%[a] 41.2%[b]	

[a]Ct cut-off value of ≤ 45 used.

[b]Ct cut-off value of ≤ 40 used.

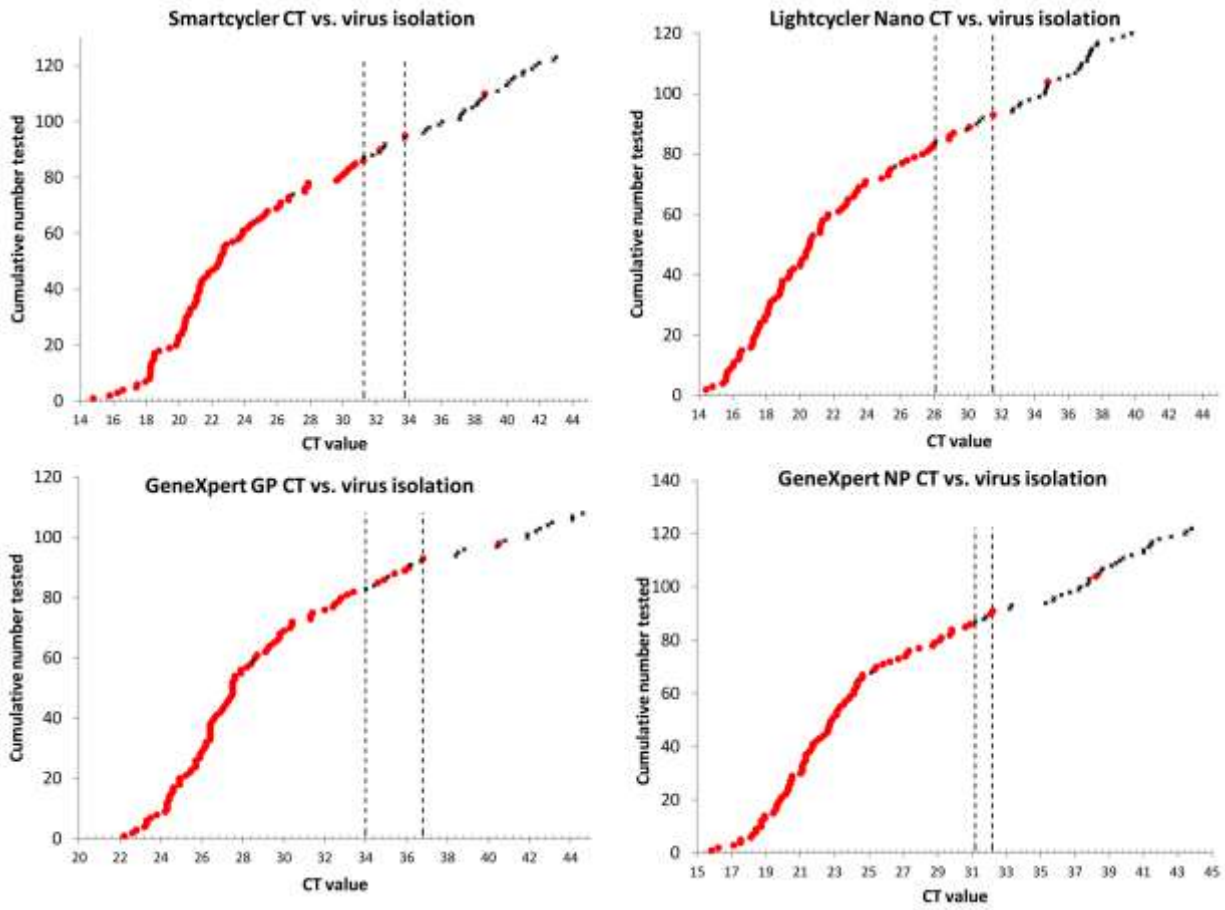

Figure 1. The range of Ct values obtained by the different assays (top left: Smartcycler; top right: Lightcycler Nano; bottom left: GeneXpert GP target; bottom right: GeneXpert NP target) and correspondence to sample infectivity is shown. Ct values are arranged in increasing value. The dotted vertical lines indicate the intermediate range where there is an overlap of Ct values corresponding to successful and unsuccessful virus isolation. Red dots indicate samples from which virus could be isolated and black crosses indicate unsuccessful virus isolation attempt. The two outliers are also included in the figures for reference.

40.5 and NP 38.2. When discarding these outliers, a relatively narrow intermediate Ct range could be established for each assay and target wherein positive and negative virus isolation results would overlap (Figure 1). For the L-gene TaqMan assay, the ranges were Ct 31.28 – 33.7 and Ct 28.08 – 31.5 on the SmartCycler and LightCycler Nano platforms respectively. On the GeneXpert the ranges were Ct 34.0 – 36.8 and Ct 31.2 – 32.2 with the GP and NP targets respectively. Excluding the outliers, values outside these ranges corresponded 100% to infectivity or non-infectivity of samples. The range of RNA copies per mL that corresponded consistently to successful virus isolation was from 9.12×10^9 to 1.33×10^5copies/mL serum (excluding the outlier) while within the range of 1.31×10^5 to 2.42×10^4 copies/mL virus could not be isolated from all the samples. Cut-off Ct values for determining the infectivity of patient samples with the different assays were determined by two-graph receiver operating characteristic (TG-ROC). The cut-off on the SmartCycler platform was determined as 31.06 Ct (yielding sensitivity of 95.45% and specificity of 97.06%). The LightCycler Nano cut-off was 28.56 (yielding sensitivity of 93.18% and specificity of 94.12%). The GeneXpert cut-off values were 34.21 (yielding sensitivity of 92.05% and specificity of 94.12%) for the GP and 31.09 (yielding sensitivity of 96.59% and specificity of 97.06%) for the NP targets.

The day of sample collection post disease onset was known for 98 of the RT-PCR positive samples (actual range day 0 – 33). Samples were arranged according to day of collection post onset in the following four groups: day 0-4 (n = 41), 5-10 (n = 42), 11-15 (n = 8) and 16-33 (n = 7). Mean Ct values and standard deviations on each assay and platform were calculated for the four time groups (Figure 2). Sample groups at earlier time points after disease onset yielded the lowest average Ct values, with an increase in time leading to higher Ct values. The percentage of samples from which Ebola virus could be recovered was highest

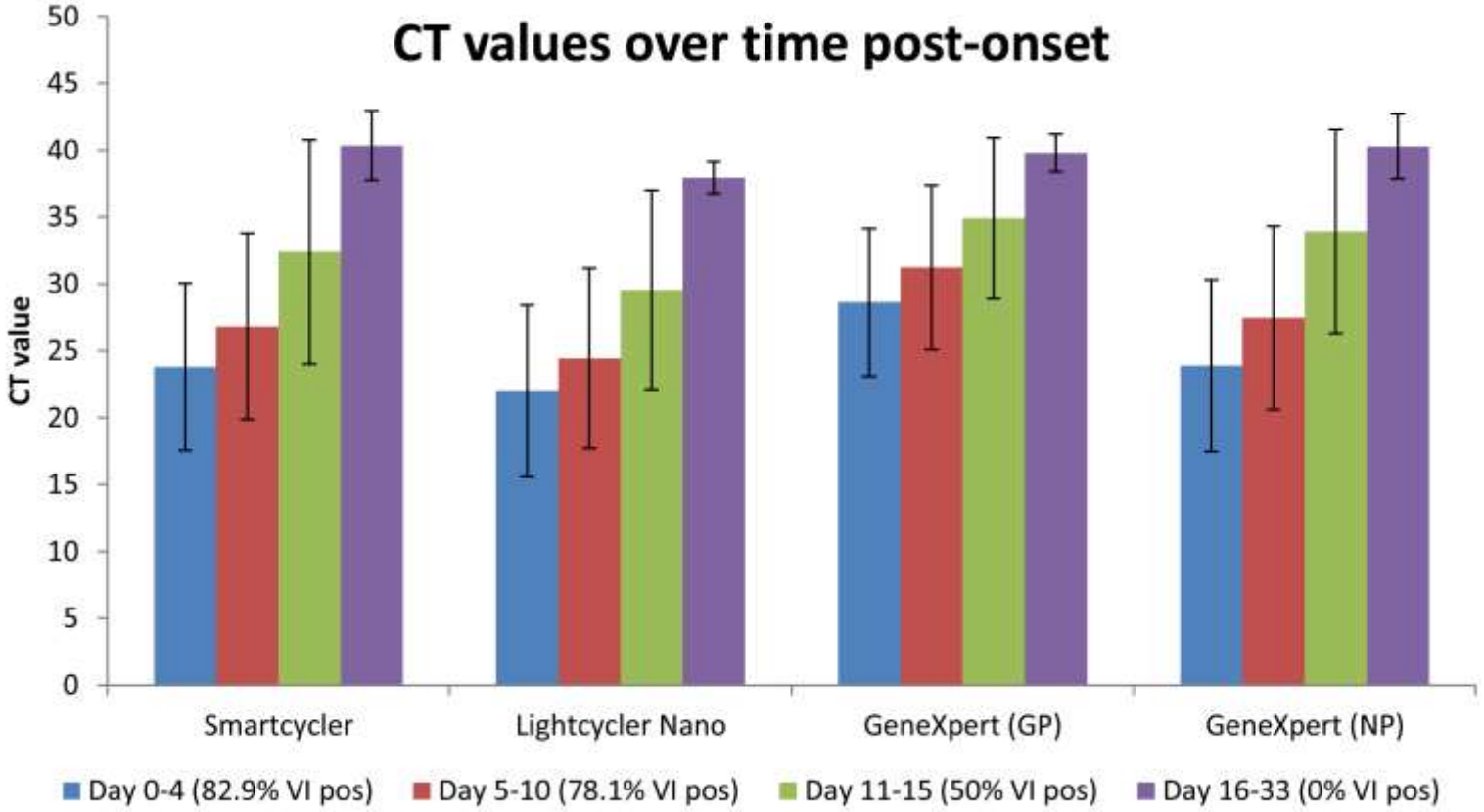

Figure 2. The average Ct values obtained by the different assays arranged according to time grouping post disease onset (blue bars: day 0-4; red bars: day 5-10; green bars: day 11-15; purple bars: day 16-33) are shown. Error bars indicate standard deviations for each time grouping. The values in the brackets below the plots indicate the percentage of samples in the time group from which Ebola virus could be isolated (VI pos). The sample numbers per time grouping are as follows: day 0-4 (n = 41); day 5-10 (n = 42); day 11-15 (n = 8); day 16-33 (n = 7).

early after onset, after which the number decreased with time, with none of the samples in the 16-33 time group containing live virus.

DISCUSSION

The magnitude of the 2013-2015 Ebola virus disease epidemic in West Africa has highlighted the unpreparedness of the world to respond to massive transmission of this highly dangerous pathogen. An important aspect of the control of such outbreaks is access to rapid and reliable diagnostic capacity, often required in remote and resource-constrained areas. Management of suspected cases in Ebola treatment or holding centres is heavily dependent on laboratory testing to ensure that infected patients are timely isolated and non-infected patients released. The availability of accurate, reliable point-of-care diagnostics would contribute greatly to better management of infected and non-infected patients, thereby decreasing the risk of unnecessary exposure of the latter. Point-of-care diagnostic capacity was lacking during most part of the West African outbreak, leading to complete dependence on mobile laboratories. The country wide transmission in each of the most affected countries and low number of laboratories performing Ebola diagnostics resulted in delays from patient submission to laboratory confirmation due to long distances, poor road infrastructure and lack of reliable sample transport.

Although real-time RT-PCR assays are currently widely used for Ebola virus diagnostics by reference laboratories worldwide and all mobile laboratories deployed in West Africa, they were not intensively validated in the field against the gold standard, the virus isolation, mostly due to limited availability of clinical specimens in the past. There is thus no standardized molecular reference test to compare and validate new prototype assays. To our knowledge this is the first evaluation of POC molecular assay in direct comparison to virus isolation in clinical specimens, and the largest clinical evaluation of currently in use real-time

RT-PCR targeting the L-gene. To illustrate the effect of PCR equipment choice, we also evaluated the performance of the L-gene real-time RT- PCR run on the Roche LightCycler Nano platform.

Analytical sensitivity and specificity of the GeneXpert Ebola assay, which targets GP and NP genes, were compared to that of a TaqMan based qRT-PCR targeting the polymerase gene, run on the Cepheid SmartCycler and Roche LightCycler Nano platforms, using a laboratory generated virus dilution series. The GeneXpert assay did not yield any cross reaction to the haemorrhagic fever viruses or arboviruses tested in this study. The limit of EBOV detection for all the assays was 1.0 $TCID_{50}$/mL (corresponding to 163 L-gene RNA copies per mL or 1.94 copies per reaction), where all four replicates at this dilution were detected. At 0.1 $TCID_{50}$/mL detection was intermittent by all assays (74 L-gene RNA copies per mL or 0.88 copies per reaction). It is important to note that detection of the GP target gene was less sensitive than detection of the NP target gene in the GeneXpert assay, with a detection limit of 1 $\log_{10}$ less. A similar trend has been observed previously, albeit in conventional PCR format, where the amount of DNA amplified by a RT-PCR targeting the L-gene was greater than that by a GP targeting assay on the same samples (12). Interestingly in the same study it was found that detection of NP was 125-fold more sensitive than detection of the L-gene, an observation we could not reproduce in this study with the lab generated virus titration series. The inherent multiplex characteristic of the GeneXpert assay might explain the difference. RT-PCR protocols following the one-step principle allows detection of both genomic and anti-genomic sense (messenger) RNA (18). Although Ebola virus has a linear, non-segmented negative sense RNA genome, inferring equal number of copies of each of the virus' genes per particle, it is possible that some genes are transcribed in higher numbers than others, leading to a higher number of detectable copies (including the mRNA) relative to other genes during infection. The number of RNA copies is not a direct

indication of the number of virus particles, with RNA copies consistently being between 3 and 4 $\log_{10}$ higher than plaque forming units (18), or between 2 and 3 $\log_{10}$ higher than tissue culture infectious dose 50 in our study.

The GeneXpert system is designed to use whole blood as sample input. It has been shown that there is earlier clearance of virus from serum and plasma than from whole blood (27). Testing of whole blood has an important practical and safety advantage since it does not require specimen processing. In our study we could only evaluate and directly compare the different assays using stored serum and plasma samples from suspected EVD cases. All assays yielded high estimates of diagnostic accuracy. As expected, when using a lower Ct cut-off value to characterize a sample as positive or negative the sensitivity decreased slightly but specificity increased. The lower specificity of the GeneXpert assay compared to the L-gene based assay might be explained by two scenarios: there is either a certain level of false positivity by the GeneXpert assay, or the GeneXpert is slightly more sensitive than the L-gene based assay. The latter is more likely considering that all of the samples that were negative by L-gene assay but positive by GeneXpert assay had Ct values above 40 on the GeneXpert assay, thus possibly representing very low positive samples. This emphasises the importance of careful interpretation of results yielding high Ct values, even above Ct 35, together with clinical data and exposure history of the patient. It appears that the performance of the GeneXpert assay is highly dependent on the detection of the NP gene, rather than GP. There were only two samples where GP gene target was detected (Ct > 40) but NP not, compared to 16 samples where NP gene target was detected (actual Ct range 38.4 to 43.8) but GP not.

Arguably a more appropriate way to directly compare performance is to look at samples grouped according to different ranges of Ct values. All samples yielding Ct values ≤ 34.99 (on the SmartCycler) were detected by all the assays (100% agreement), with

agreement decreasing in lower positive samples. The value of detecting both NP and GP in the GeneXpert assay is illustrated when analysing the results at Ct range 40-45. When detection of GP and NP is analysed separately at this range, the agreement to the L-gene assay is 20% and 90%, respectively. However, when following the principle of regarding a sample as positive with detection of either GP or NP or both, the agreement becomes 100%. Another important analysis was to demonstrate that the assays are reliable for the detection of RNA in samples that contain live virus. All assays were able to detect RNA in samples that tested positive by virus isolation. As expected, all the assays detected RNA in a number of samples that did not contain live virus. Similarly to what was reported for Marburg virus in bats (28), we found a clear correlation between Ct values (RNA copies), and the ability to isolate virus from the clinical specimens. Marburg virus could only be isolated from bat tissues which yielded Ct values lower than 35 (28). In our own study we could also not isolate EBOV from blood and tissues of experimentally infected bats with Ct values lower than 35 (NICD unpublished data).

A direct correlation between viral RNA levels determined by q-RT-PCR and the ability to isolate EBOV carries potentially important practical aspects in terms of identifying infectious or non-infectious samples. In our study we could identify an intermediate range where there was an overlap of Ct values yielding positive virus isolation and not, and this Ct range was different for the different assays. Excluding the limited outliers, all the assays perform 100% at Ct values below 35 and all are able to detect RNA in samples from which virus could be isolated.

Although we do not have matching serology data available, another explanation for the detection of RNA in virus isolation negative samples might be that the RNA detected in the negative isolation samples represent virus that is in the process of being cleared in the form of immunocomplexes. This might be supported by the fact that percentage of successful

virus isolation decreased over time post-onset (Figure 2). Considering that, there are numerous factors that can have an effect on the outcome of a diagnostic test on a patient's clinical sample. Sample quality is an obvious factor which includes various aspects such as volume, haemolysis, cold-chain transport and storage. Probably a more important factor is the timing of sample collection post disease onset. A sample collected too early or too late after disease onset can yield a false-negative result depending on which analyte is targeted. The duration of viremia caused by infection with different viruses differs, and also the time needed to develop a detectable antibody response. For this reason the diagnosis of a viral haemorrhagic fever should ideally not depend on a single test result (single analyte), especially in the case of excluding VHF as a diagnosis.

In this study we showed that the prototype GeneXpert Ebola assay was highly accurate in detection of RNA in serum and plasma samples containing live Ebola virus. The agreement between the different assays we compared was very high at low Ct values, and decreased with lower positive samples. Despite the sample input volume of the GeneXpert assay being only 100 µL compared to 140 µL used for manual RNA extraction, it did not affect assay sensitivity noticeably. The prototype GeneXpert Ebola assay presents several advantages over currently available qRT-PCR protocols. The assay incorporates an automated extraction and sample addition process, making it possible to be run by minimally trained technicians within 90 minutes. If placed within Ebola treatment centres, the GeneXpert system would negate the need for additional biocontainment devices where patient samples first have to be inactivated, processed and RNA extracted. The technology minimises possibility of human error, for example during the extraction process, RT-PCR master mix preparation and sample addition, by being automated. One other advantage of the assay is the usage of stable reagents. The ongoing Cepheid shelf life testing demonstrates that cartridges used for Xpert

Ebola POC are stable under room temperature at least for 6 months (Cepheid, personal communication).

Haemoglobin and lactoferrin have been identified as major inhibitors of diagnostic PCR in human blood cells (29). However, an internal control included in the Ebola Xpert POC ensures the detection of inhibitory effects from factors possibly present in patient samples.

False negative results in patients with severe haemorrhagic fever have been noted before (30). Therefore, analytical results obtained by this and any other test should be interpreted by trained and experienced diagnosticians together with all available laboratory results, clinical, pathological, and epidemiological data to ensure accurate diagnosis. In conclusion, the prototype GeneXpert Ebola assay represents a promising point-of-care screening tool to make rapid presumptive decisions about patient management and infection control measures.

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AUTHOR CONTRIBUTIONS

JTP, PJvV, IS and AK conceived and designed the study. PJvV, NS, OC, KK and JTP were responsible for sample processing, long-term storage and database management in Sierra Leone. PJvV, NS and AG executed the laboratory work. PJvV, AG and JTP interpreted and analysed the data. PJvV drafted the manuscript after which it was critically reviewed by all co-authors.

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