

Detection of dengue virus in samples from suspected yellow fever cases in Ghana

ABSTRACT

Background: Dengue fever remains a serious public health treat throughout the world. Ghana shares borders with countries that have reported dengue cases, yet no case has been reported in Ghana. Dengue infections with its broad range of clinical presentations make it potentially unrecognized by clinicians. In this study, serological tests were used to detect antigens and antibodies and molecular tests were used to detect viral RNA specific to dengue viruses in archived human serum specimens in Ghana.

Methods: Blood samples of 360 patients aged 6 months to 82 years old with suspected yellow fever in hospitals across Ghana were tested for dengue virus exposure. Samples were screened using SD Dengue NS1 Ag ELISA and the SD Dengue IgM/ IgG capture ELISA test, which detects dengue virus antigen and antibodies to dengue virus in human blood.

Results: A total of 360 serum samples were tested, 8% (29/360) were found positive by the antigen/antibody ELISA test, 2.5% (9/360) were positive by NS1 ELISA with 1.9% (7/360) and 3.6% (13/360) being positive by IgM and IgG ELISA respectively. None of the specimens tested positive by real-time RT-PCR. 0.6% (2/360) of cases tested positive for both NS1 and IgG. The proportion of NS1 antigen positivity was highest in the Brong Ahafo region 0.33 (3/9) followed by Ashanti and Upper West regions with 0.22 (2/9) respectively. IgM positivity was, however, highest in Upper West 0.43 (3/7).

Conclusion: Introduction of dengue virus surveillance with routine diagnosis will serve to alert the health system of the possible outbreak and also minimize spread in the event of an outbreak in the country.

Key words: Dengue virus, Yellow fever virus, Sero-prevalence, ELISA, Ghana

INTRODUCTION

Dengue is an acute viral illness caused by the dengue virus which is made up of four closely related but antigenically distinct serotypes (Dengue 1-4) [1]. All four Dengue virus (DENVs) serotypes circulate in South America, where almost all countries are hyper-endemic for DENV transmission [2]. In 2014, the World Health Organization (WHO) estimated that 50-100 million human DENV infections occur annually in tropical and sub-tropical regions, posing considerable public health problems in over 100 countries [3]. In Africa DENV was first isolated in Ibadan, Nigeria in 1960 [4] and since then, the disease has been reported in Ivory Coast, Burkina Faso and Nigeria [5]. Recently in 2009, a large outbreak of dengue involving over 17,000 cases was documented in the Cape Verde islands [6]. DENV importation from West African countries to European countries have also been documented [7, 8, 9]

Dengue fever is self-limiting though debilitating febrile illness whose symptoms include fever, headache, muscle and joint pains, and a characteristic skin rash that is similar to measles [37] In

some cases, disease develops into life-threatening dengue hemorrhagic fever or Dengue shock syndrome and death [10, 3].

The DENV is a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family and are transmitted to humans by virus-carrying *Aedes* mosquitoes; the same type of mosquitoes implicated in yellow fever transmission [3, 11]. The *Aedes spp* of mosquitoes is found in close association with human settlements throughout the tropics, breeding mainly in containers in and around buildings, car tyres and water-filled tree-holes [12, 13].

Dengue fever is rarely fatal and survivors appear to have lifelong immunity to the homologous serotype. The mortality from DHF can exceed 30% if appropriate care is unavailable. The most significant risk factor for DHF is when secondary infection with a different serotype occurs in people who have already had and recovered from, a primary dengue infection. It is suggested that virus infection is enhanced by the presence of pre-existing heterotypic antibodies [14, 15].

There is no cure for Dengue fever or for DHF. Currently, the only treatment is the management of symptoms which is known to reduce mortality from DHF to less than 1% [16].

The non-structural protein 1 (NS1) encoded in the viral genome has been shown to be useful as a tool for the diagnosis of acute dengue infections [17, 18]. Dengue NS1 antigen has been detected in the serum of DENV infected patients as early as 1-days post onset of symptoms (DPO), and up to 18 DPO. The NS1 antigen is useful for differential diagnosis between flaviviruses because it is highly specific for dengue [19]. Conversely, dengue IgM and IgG antibodies which are used to determine recent and past dengue infections, are prone to cross-reactivity with other members of the *Flavivirus* genus such as West Nile Virus, Japanese Encephalitis Virus and Yellow Fever Virus [20, 21].

There has been no documented case of dengue virus infection in Ghana although Ghana shares common borders with Ivory Coast and Burkina Faso [22, 23], where Dengue cases have been detected from an outbreak [24]. A research study involving 218 children positive for malaria in Ghana showed that 21.6% had previous exposure to dengue while 3.2% had recent exposure [25]. An international expert conference on Dengue in Africa was held in Accra, Ghana, in 2013 to consider why dengue in Africa does not cause a disease incidence of a magnitude similar to that of Latin America, or why dengue fever is not spreading to Africa for epidemiological reasons still not understood. The report suggests, among other research, retrospective studies to be conducted for dengue virus in serum samples stored in repositories tested for yellow fever. These results can be used to clarify the extent of dengue transmission in Africa [26].

Currently, there are surveillance activities on yellow fever in Ghana but very little is being done on dengue. Although many samples of suspected yellow fever cases when screened turn out to be negative in the laboratory, such samples are not investigated for other likely endemic arboviruses such as Dengue and Chikungunya virus. This study investigated the presence of antigens and antibodies specific to dengue viruses in archived human serum specimens of suspected yellow fever cases in Ghana, using both serological and molecular methods. We also matched these diagnostic markers of Dengue infection to areas where they might be significantly prevalent.

MATERIALS AND METHODS

Study population and sample collection

This was a laboratory based cross-sectional study which involved the use of archived samples from patients suspected of yellow fever infection at the National Public Health and Reference

Laboratory (NPHRL) in Accra. The NPHRL of the Ghana Health Service is a World Health Organization (WHO) designated and accredited laboratory for the testing of all suspected yellow fever cases in Ghana. The laboratory receives clinical specimens of serum or plasma from suspected yellow fever cases across the country through the Disease Surveillance Department (DSD) for epidemic prone diseases or diseases of public health importance. We tested 360 archived serum samples collected between January and December 2013 from all the ten regions of Ghana, stored at -80°C for the presence of Dengue NS1 antigen as well as dengue IgM/ IgG using anti-Dengue specific IgM, anti-Dengue specific IgG ELISA test kits. These archived samples had previously tested negative for anti-yellow fever specific IgM antibodies using the sandwich ELISA protocol developed by WHO. Subsequently, all ELISA positive Dengue samples were sent to the Noguchi Memorial Institute for Medical Research for molecular testing. Demographic and epidemiological data was obtained from the case-based forms that accompanied the clinical specimens.

Serologic Testing for Dengue Infection

Enzyme-Linked Immunosorbent Assay (ELISA) for the qualitative determination of antigens and antibodies specific to dengue virus was performed using the SD Dengue NS1 Ag ELISA and the SD Dengue IgM/IgG capture ELISA as described by the manufacturer, SD Bioline. Subsequently all positive samples were serologically confirmed using SD Bioline Dengue Duo rapid test kit with NS1, IgM and IgG combo test.

Nucleic acid isolation and purification

Twenty-nine (29) clinical specimens that tested positive with SD Dengue NS1 Ag ELISA and or the SD Dengue IgM/IgG capture ELISA were selected for molecular analysis. Ribonucleic acids (RNA) were extracted and purified from serum and/or plasma specimens with the QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) in accordance with the manufacturer's instruction with slight modifications. Briefly, 140µl and 70µl of sera/plasma from each pool was separately mixed with 560µl of a lysis buffer, AVL from the kit. After an incubation of 10 min, 500µl non-denatured absolute ethanol (100%) was added and the RNA was affinity-purified as described in the manufacturer's instructions of the kit. The purified RNA was eluted in 60µl of buffer AVE equilibrated to room temperature from the kit.

Detection by Real time Reverse Transcription-Polymerase Chain Reaction

Volumes each of 5µl of the extracted and purified RNA duplicates from the pooled clinical sera or plasma were used in a TaqMan assay real time reverse transcription-polymerase chain reaction (rRT-PCR) assay using the Applied Biosystems® 7300 RT-PCR system (Life Technologies, Grand Island, NY, USA). In singleplex reaction mixtures, the RNA was combined with 10pmol each of Dengue serotype specific primer and probe sets with targets describe by Johnson B.W *et al.*, 2005 in a 25-µl reaction. Each reaction mixture contained a single dengue serotype primer pair and probe – making eight separate reactions for the isolated and purified RNAs for each clinical specimen. The reagent master mix was prepared according to the number of reactions required for each test run using the AgPath-ID™ One-Step RT-PCR kit (#AM1005, Thermo Fisher Scientific, NY, USA). The master mix was divided and dispensed into portions of 20 µl each into an optical-ready 96-well plate (MicroAmp® Optical 96-well reaction plate,

Thermo Fisher Scientific, NY, USA). Then a final addition of 5µl of individual RNA to each appropriately labeled well. Included in each test run were 'no RNA' or water reagent and dengue positive RNA controls.

Result interpretation was in accordance with the instructions of the software (SDS version 1.4 patch 2) used with the Applied Biosystems® 7300 RT-PCR system (Life Technologies, Grand Island, NY, USA). Test runs that exhibited fluorescence growth curves that cross or did not cross the threshold line for 'no RNA' or positive control reagent reactions respectively were invalidated and repeated. A presumptive positive for Dengue serotype specific virus was considered for a clinical specimen when the controls met stated requirements and the growth curves crossed the threshold line within 40 cycles with the inverse true for negatively considered clinical specimens for dengue serotype specific virus. Test runs were valid, however none of the pooled extracts was detected to be positive - had growth curves that crossed the threshold line with the exception of the positive controls.

Conventional RT-PCR

Published primers by Lanciotti et al., 1992 were used to perform a conventional RT-PCR on the pooled clinical specimens with slight modifications with the use of the thermal cycler, (Aeris G-96 well by Esco Micro Pte Ltd, Singapore). Total reaction volumes of 25µl including 5µl of the target RNA from the Qiagen viral RNA mini kit (QIAGEN, Hilden Germany). The RT-PCR (45 cycles) contained QIAGEN OneStep RT-PCR reagents with the sense primer Duc 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3' homologous to the genomic RNA of the four serotypes and the antisense primer Dus 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3', as

described in detail by Lanciotti et al., 1992. The reactions were proceeded for 30 minutes at 50°C and then 95°C for 15 minutes for initial denaturation followed by 40 cycles of denaturation [(94°C for 30 seconds); primer annealing (55°C for 30 seconds); and primer extension (72°C for 1 minute) along with final extension (72°C for 10 minutes)]. The amplification products (expected size, 511 base pairs) were electrophoresed on a 2% Agarose gel (peqlab Biotechnologie, Erlangen, Germany), stained with Ethidium bromide, and viewed under UV light.

Data Analysis

Data were double entered and cleaned in Excel and the analyses done with RStudio (Version 0.99.878) and Epi Info software package version 3.5.1. Appropriate measures of central tendency for mean, median, frequency distributions, percentages, and standard deviation were calculated.

Ethical considerations

Human sera samples analysed in the study were initially collected with the national ethical guidelines as part of the national yellow fever surveillance programme in Ghana and sent to the National Public Health and Reference Laboratory in Accra, Ghana. This does not require ethical clearance. Since yellow fever was negative, the laboratory wanted to ascertain whether Dengue virus is the ethiologic agent of the suspected case. We sought permission from the Disease Surveillance Department to use their data. We protected the confidentiality of participants

through use of codes. However, ethical committee review did not apply as this was a public health response to surveillance. The study does not involve the use of animals or animal samples.

RESULTS

Of the 360 archived samples processed, 44% (159/360) and 56% (201/360) were from females and males respectively. One percent of the study sample (4/360) was positive for yellow fever.

The proportion of cases that were positive for Dengue NS1 antigen was 2.5% (9/360). For Dengue IgM, 1.9% of cases were positive while 3.6% of cases were positive for Dengue IgG (Table).

The age of the population ranged from seven months to 82 years with median age of 16 years, mean age of 19.2 and standard deviation of 16.1. The median age for females was 16 years and that for males was 15 years. Median age for cases that were positive for dengue NS1 antigen, dengue IgM and dengue IgG was 27 years, 22 years and 15 years respectively. Table2 presents summary statistics on age grouped by variables of interest.

All the samples that were found positive by NS1 antigen test and capture ELISA for both anti-IgM and anti-IgG antibodies to Dengue were further subjected to molecular testing but all were found to be negative by RT-PCR.

Most study patients, 174 (48.3%), were 15 years or younger. The results show that dengue NS1 antigen was relatively more prevalent (N=4) aged 16-30 years. In addition, 6 out of the 9 positive cases for Dengue NS1 antigen were 30 years or younger.

Of the 7 positive cases for dengue IgM antibodies, 6 were detected among the age groups of 45 years or less.

Although no Dengue NS1 was detected in cases older than 75 years, anti-IgM was detected in 1 case in this age group.

The positive Dengue IgG was relatively more prevalent in the range of 15 or less (N = 7).

Neither anti-IgM nor anti-IgG were detected in cases between 61-75 years, although the NS1 antigen has been detected in this age group (Table 2).

The results presented in Table indicate that none of the samples included in this study was positive for both Dengue NS1 antigen and Dengue IgM. However, 2 out of 360 cases tested were positive for both NS1 and IgG. In addition, 7/360 of the samples that were positive for Dengue IgM were negative for the NS1 antigen and 11/360 were positive for Dengue IgG and negative for NS1 antigen.

As shown in Table 4, most of the samples in this study, 21% (74/360), came from the Brong Ahafo region while the smaller sample came from the Northern region, 2% (8/360). The largest proportion of the samples which showed NS1 antigen positivity also came from the Brong Ahafo region (0.33) followed by Ashanti and Upper West regions with 0.22 respectively. The proportion of positive IgM was higher in Upper West (0.43). The IgM test detected no cases in Brong Ahafo (Table). On the other hand, the Eastern region recorded the highest IgG positivity index (0.31). Both Ashanti and Volta region recorded relatively high proportions of IgG, 0.23 and 0.15 respectively (Figure 1).

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215 **DISCUSSION**

216 Dengue has long been thought to be widespread in Africa but case reporting remains very poor.
217 In the absence of laboratory test facilities, it is likely that Dengue fever, as opposed to DHF,
218 would often be mistaken for other diseases such as malaria [25, 27, 28]. In this study we
219 employed serological and molecular assays for the identification of probable dengue cases in
220 yellow fever negative samples. A strategy aimed at increasing the capacity for surveillance and
221 outbreak response, using early and accurate diagnosis was adopted. We were able to identify
222 NS1 Ag in 2.5%, anti-IgM antibodies in 1.9% and anti-IgG antibodies in 3.6% of all the study
223 samples with the serological assay but none was confirmed with the molecular tools of RT-PCR
224 used in the present study. Even though the overall sensitivity based on this test is quite low, it is
225 consistent with a study in Pakistan [29] but different from another study to evaluate an enzyme
226 immunoassay for detection of Dengue virus [30] that recorded higher sensitivity. The low
227 positivity could be that the samples were collected from patients with suspected yellow fever
228 which may have other infections that clinically resemble Dengue and may not meet the case
229 definition for dengue [31]. Those patients whose samples tested positive for anti-Dengue specific
230 IgM antibodies by IgM antibody capture ELISA assay but were negative by RT-PCR result in
231 the acute phase specimen, may be classified as having had recent probable Dengue infection.
232 This is due to the fact that IgM antibodies for Dengue may remain elevated in serum for 2 to 3
233 months after the illness [32].

234 In the present study, we found Dengue to be more in males than females and infection was found
235 in all the age groups except for those 75 years and above. The highest number of positive cases

236 of NS1 between 16 and 30 years of age observed correlated with the results of a study conducted
237 by Halstead [10, 30], Neeraja in India [34] and Khan in the Indo-Myanmar border area [35].
238 Although tests which detect antibodies as markers of Dengue infection may cross-react with
239 other members of the Flavivirus family, the detection of the NS1 antigen in some of the study
240 samples is a sure sign of Dengue infection. This is because the NS1 antigen is highly specific for
241 Dengue [36]. According to our results some NS1 negative cases were found positive for IgM
242 antibodies and some IgM negative cases were found positive for NS1 antigen; these results are in
243 agreement with studies conducted by Dussart et al., 2006 [31] and Peeling et al., 2010 [37].
244 Furthermore, 2 cases tested positive for both NS1 antigen and IgG antibodies. Therefore, these
245 reciprocal results from both assays (NS1 and IgM ELISA) showed that the overall diagnostic
246 capability to identify DENV infections can be increased by using a combination of these tests.
247 Judging from the dynamics of immune responses to Dengue infection, we can speculate that the
248 two samples which tested positive for both dengue NS1 antigen and Dengue IgG antibodies, had
249 been exposed to a secondary dengue infection.

250 Although the Brong Ahafo and Northern regions are contiguous regions and the two largest
251 regions by land size, one (Brong Ahafo) presented the largest number of suspected cases while
252 the Northern region presented the least. Similarly, the greater proportion of NS1 antigen
253 positivity came from the Brong Ahafo region while the Northern region showed zero positivity.

254 Our study could not confirm the ELISA positive cases by RT-PCR, hence could not distinguish
255 the Dengue serotypes. This is the study limitation. However, the detection of NS1 antigen and
256 IgM antibodies is an indication of exposure and possible circulation of Dengue virus. Dengue is
257 likely being missed in Ghana because of low awareness by health care providers, prevalent

febrile illnesses, and lack of diagnostic testing and systematic surveillance. Other hypotheses to explain low reported numbers of cases include cross-protection from other endemic flavivirus infections, genetic host factors protecting against infection or disease, and low vector competence and transmission efficiency. However, it is still unclear why the circulation of dengue virus in Africa and this includes Ghana, does not result in serious cases such as in Asia and Latin America [26]. The correct detection of the circulation of dengue virus may pave the way for studies that indicate solutions on how to deal with the severe manifestations of dengue in other regions of the world. It is noteworthy positive tests for yellow fever virus in samples archived as negative for this virus. This result underscores the need to invest and provide more sophisticated diagnostic tools for health services.

CONCLUSION

Dengue disease appear to be in Ghana at least since 2013. Silent circulation of different Dengue serotypes may predispose infected individuals to Dengue hemorrhagic fever in infection by a different serotype. Active surveillance with close monitoring of cases, coupled with training of clinicians on case detection in the health facilities in Ghana is essential to understand the dynamics and disease burden of Dengue. Further studies are needed to elucidate the transmission and non-detection of dengue in Ghana and the possible epidemiological implications for the population of Ghana and visitors.

LIST OF ABBREVIATIONS

IgM = Immunoglobulin M; IgG – Immunoglobulin G; ELISA – Enzyme-Linked Immunosorbent assay; Ag – Antigen; YF = Yellow Fever; DENV = Dengue virus; DHF = Dengue hemorrhagic fever; DPO = Days Post Onset; NPHRL – National Public Health and Reference Laboratory; WHO = World Health Organization; RNA = Ribonucleic acid; RT-PCR = Reverse Transcriptase Polymerase Chain Reaction.

DECLARATIONS

We retrieved stored sera samples and analysed for Dengue virus in the study according to the national ethical guidelines as part of the National yellow fever surveillance programme in Ghana and sent to the National Public Health and Reference Laboratory in Accra, Ghana to ascertain whether Dengue virus is the etiologic agent of the diagnosed yellow fever. We sought permission from the Disease Surveillance Department to use their data. We protected the confidentiality of participants through use of codes. However, ethical committee review did not apply as this was a public health response to surveillance. The study does not involve the use of animals or animal samples

Ethical approval and consent to participate; this section is not applicable

Consent for publication; this section is not applicable

298 **Competing interests**

299 None declared.

300

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304 **Author's contributions**

305 LHOA participated in the study design, sample processing, analysis of results and manuscript
306 writing, RK was involved in analysis of results and writing of manuscript, BA was involved in
307 sample processing and analysis of results, JHKB was involved in sample processing, data
308 interpretation and editing of the manuscript, GB participated in data analysis and editing of the
309 manuscript, RBA was involved in data analysis and manuscript writing, DO was involved in the
310 writing of manuscript, JKO supervised the study protocol, analysis, editing and interpretation of
311 data and manuscript writing. All authors have read and approved for submission.

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Table1: Descriptive statistics of results according to gender and test performed on 360 blood samples

Variable	N	N%
Sex		
Female	159	44.2
Male	201	55.8
Dengue NS1.Ag		
Negative	351	97.5
Positive	9	2.5
Dengue IgM		
Negative	353	98.1
Positive	7	1.9
Dengue IgG		
Negative	347	96.4
Positive	13	3.6
YF IgM results		
Positive	4	1.1
Negative	352	97.8
Equivocal	1	0.3

443 **Table2:** Distribution of number of negative and positive results by age group

Age Categories							
Variables	0-15yr	16-30yr	31-45yr	46-60yr	61-75yr	Above 75yr	Total
Dengue NS1 Results							
Negative	172	98	54	16	6	5	351
Positive	2	4	1	1	1	0	9
Dengue IgM Results							
Negative	172	100	53	17	7	4	353
Positive	2	2	2	0	0	1	7
Dengue IgG Results							
Negative	167	99	53	16	7	5	347
Positive	7	3	2	1	0	0	13
Total	174	102	55	17	7	5	360

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446 **Table3:** Cross tabulation of dengue NS1 antigen results against dengue IgM and IgG results respectively

Dengue NS1 Results			
	Negative	Positive	Total
Dengue IgM Results			
Negative	344	9	353
Positive	7	0	7
Dengue IgG Results			
Negative	340	7	347
Positive	11	2	13
Total	351	9	360

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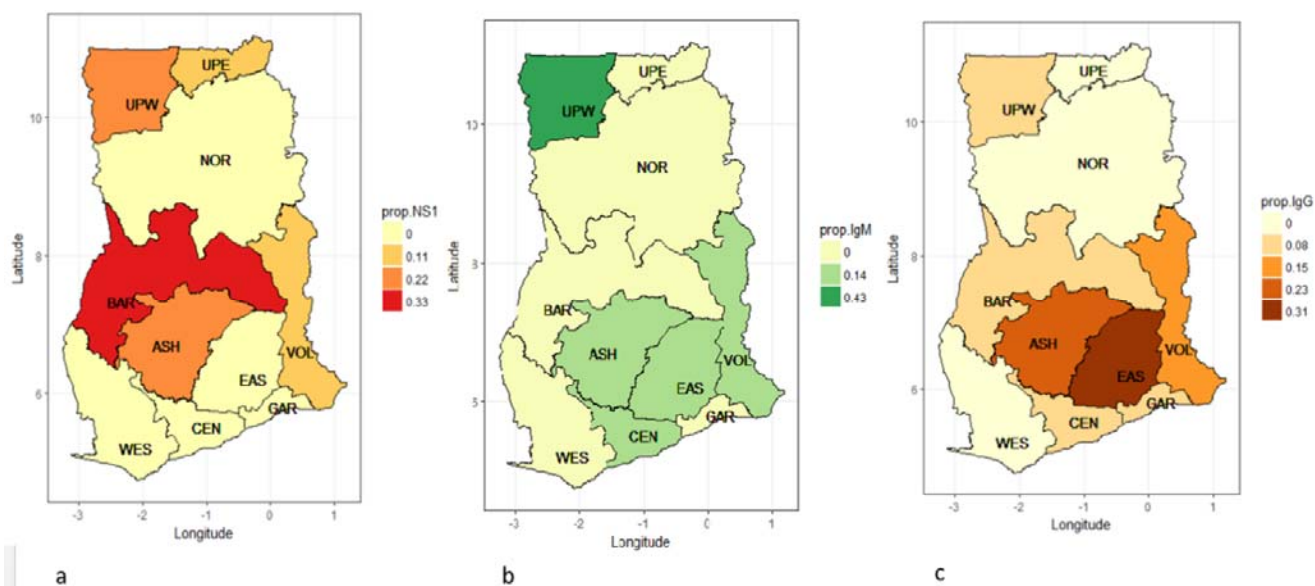
448

449 **Table4:** Distribution of positive and negative numbers by test and region in Ghana

Dengue NS1 Results		Dengue IgM Results		Dengue IgG Results		Total Positive
Region	Negative	Positive	Negative	Positive	Negative	

Ashanti	48	2 (0.22)*	49	1 (0.14)*	47	3 (0.23)*	06
Brong Ahafo	71	3 (0.33)	74	0	73	1 (0.08)	04
Central	9	0	8	1 (0.14)	8	1 (0.08)	02
Eastern	60	0	59	1 (0.14)	56	4 (0.31)	05
Greater Accra	11	0	11	0	10	1 (0.08)	01
Northern	8	0	8	0	8	0	00
Upper East	29	1 (0.11)	30	0	30	0	01
Upper West	60	2 (0.22)	59	3 (0.43)	61	1 (0.08)	06
Volta	35	1 (0.11)	35 (9.7)	1 (0.14)	34	2 (0.15)	04
Western	20	0	20 (5.6)	0	20	0	00
Total	351	9	353	7	347	13	29

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452 **Figure1: Geographical distribution of positive results for dengue second test in Ghana: a) dengue NS1 antigen,**
453 **b) dengue specific IgM antibodies and c) dengue specific IgG antibodies.**