Table 1. List of primers used in HIV-1 genotypic drug-resistance testing.

Target region	Amplicon		Primers			
	Size (bp)	Position[a]	Reaction	Direction	Name	Nucleotide sequence (5′ to 3′)
Protease	424	2,168 to 2,591	RT-PCR	Forward	DRPRO5	AGA CAG GYT AAT TTT TTA GGG A
				Reverse	DRPRO2L	TAT GGA TTT TCA GGC CCA ATT TTT GA
			Nested PCR	Forward	DRPRO1M	AGA GCC AAC AGC CCC ACC AG
				Reverse	DRPRO6	ACT TTT GGG CCA TCC ATT CC
Reverse transcriptase	838	2,510 to 3,347	RT-PCR	Forward	DRRT1L	ATG ATA GGG GGA ATT GGA GGT TT
				Reverse	DRRT4L	TAC TTC TGT TAG TGC TTT GGT TCC
			Nested PCR	Forward	DRRT7L	GAC CTA CAC CTG TCA ACA TAA TTG G
				Reverse	DRRT6L	TAA TCC CTG CAT AAA TCT GAC TTG C

bp, base pairs; PCR, polymerase chain reaction; and RT-PCR, reverse transcription and polymerase chain reaction.
[a]Amplicon positions in the reference HIV-1 HXB2 sequence are represented.
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Results

CRF02_AG is the Predominant HIV-1 Strain in Koforidua, Ghana

During the study period, 101 HIV-1-infected patients were enrolled in this study. As shown in Table 2, 90 cases were adults (≧15 years old), including 59 newly diagnosed ART-naïve cases and 31 ART-experienced cases. The remaining 11 cases were children (<15 years old), among which were 10 newly diagnosed ART-naïve cases while one child was ART-experienced (Table 3). To understand the molecular epidemiology of HIV-1 infections in Ghana, we analyzed the *pol* gene sequences in detail through the construction of phylogenetic trees, similarity plotting, and bootscanning analyses. Among the 101 cases, 75 (74.3%) were identified as HIV-1 subtypes and CRFs (Fig. 1A); 67 were CRF02_AG (66.3%), 4 were sub-subtype A3 (4.0%), 2 were CRF06_cpx (2.0%), and 2 were CRF09_cpx (2.0%). Thus, our analyses clearly showed the predominance of HIV-1 CRF02_AG in Koforidua.

Interestingly, the remaining 26 cases (25.7%) were identified as HIV-1 URFs (Fig. 1B). The most prevalent chimeric pattern was CRF02/A3 ($n=11$, 10.9%), followed by CRF02/CRF06 ($n=5$, 5.0%), CRF02/G ($n=2$, 2.0%), CRF02/CRF09 ($n=2$, 2.0%), and 6 other patterns (Fig. 1C). Of note, two interesting clusters were found in the phylogenetic tree of URF isolates (Fig. 1B). Cluster #1 with three isolates, 09GH.120495, 09GH.1765, and 09GH.KF47, shared the same mosaic *pol* gene comprising a large PR and RT fragment of CRF02 and a short RT fragment of A3 (cluster #1, Fig. 1B). Cluster #2 with the other three isolates, 09GH.107421, 09GH.60390, and 09GH.KF43, shared the same mosaic *pol* gene comprising the PR fragment of A3 and two RT fragments of CRF02 and A3 (cluster #2, Fig. 1B). Our data suggest that the two URF clusters are candidates for a new CRF spreading in this area of Ghana.

HIV-1 Drug-resistance Mutations are Highly Frequent among ART-experienced Cases with Virological Failure Status

Demographic and clinical characteristics of 31 ART-experienced adult cases are shown in Table 2. All patients except one (96.8%, 30/31) were treated with the first-line ART regimen of 2 NRTIs+NNRTI, and the remaining one (3.2%) with the second-line ART regimen of 2 NRTIs+PI. Their median duration of ART was 16.1 months (IQR, 6.8–30.3 months), and most cases maintained their adherence at a "good" or "satisfactory" level (80.6%, 25/31).

Among these ART-experienced adult cases, 22 cases (71.0%) possessed one or more HIV-1 drug-resistance mutations (Table 4). The most prevalent drug-resistance pattern was 2-class resistance to NRTI and NNRTI ($n=13$, 41.9%), followed by 1-class resistance to NNRTI ($n=8$, 25.8%). Of note, 3-class resistance was identified in one case (3.2%) treated with the second-line regimen AZT+3TC+NFV. This case possessed HIV-1 RT mutations M41L, V90I, A98G, M184V and T215Y, and the major NFV-resistance mutation L90M in PR. As shown in Table 4, the most prevalent drug-resistance mutation among the 31 cases was M184V ($n=12$, 38.7%), followed by K103N ($n=9$, 29.0%), and T215Y/F ($n=6$, 19.4%). No drug-resistance mutation was detected in the remaining 9 cases (29.0%, Table 4), suggesting that acquisitions of drug resistance was not the primary cause of their virological failure. The cases with and without resistance did not differ significantly in their demographic characteristics.

Furthermore, we analyzed the chronological order of acquiring drug resistance to 3TC, NVP, EFV, AZT, and d4T. As shown in Fig. 2A, no mutation was found in any patients, even with viremia, who had received ART for ≦6.0 months (0%, 0/6). However, M184V mutation was detected in 37.5% (3/8) of patients with 6.1–12.0 months of ART, and the prevalence increased to 80.0% (4/5) at ≧36.1 months of ART (red bars in Fig. 2A and 2B). In the case of NVP and EFV resistance, K103N, V106A, V108I, Y181C/L, G190A, P225H, and M230L mutations were detected in more than half of patients after 6.0 months of ART (blue bars in Fig. 2A and 2B). Importantly, the prevalence and accumulation of thymidine analog-associated mutations (TAMs) appeared to be higher with longer duration of ART; 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months of ART (green bars in Fig. 2A and 2B).

Low HIV-1 Drug-resistance Transmission in ART-naïve Cases

The general demographics of the 59 adult ART-naïve cases were similar to those of the treated cases, however pVL was significantly higher in the naïve cases ($P=0.006$) (Table 2). Among the ART-naïve cases, no transmitted HIV-1 drug-resistance mutation was found (Table 4). However, polymorphisms at

Table 2. Demographic and clinical characteristics of ART-experienced and -naïve HIV-1-infected patients ≧15 years old ($n=90$).

Characteristic		ART-experienced, $n=31$	ART-naïve, $n=59$	P
Age, years	Median (IQR)	38 (35–43)	42 (32–49)	0.251
Sex (%)	Female	18 (58.1)	38 (64.4)	0.649
	Male	13 (41.9)	21 (35.6)	
Risk factor for HIV infection (%)	Heterosexual contact	31 (100.0)	57 (96.6)	0.543
	Transfusion	0 (0.0)	2 (3.4)	
HIV serology (%)[a]	HIV-1 positive	29 (93.5)	59 (100.0)	0.116
	HIV-1 and -2 positive	2 (6.5)	0 (0.0)	
$CD4^+$ T-cell count, cells/μl	Median (IQR)	230 (87–376)	237 (86–426)	0.750
HIV-1 viral load, log_{10} copies/ml	Median (IQR)	3.7 (3.3–4.1)	4.4 (3.7–5.0)	0.006
HIV-1 genotype (%)	CFR02_AG	18 (58.1)	42 (71.2)	0.638
	A3	2 (6.5)	2 (3.4)	
	CRF06_cpx	1 (3.2)	1 (1.7)	
	CRF09_cpx	1 (3.2)	1 (1.7)	
	URF	9 (29.0)	13 (22.0)	
ART regimen (%)				–
First line	d4T+3TC+EFV	10 (32.3)	–	
	AZT+3TC+NVP	10 (32.3)	–	
	AZT+3TC+EFV	7 (22.6)	–	
	d4T+3TC+NVP	3 (9.7)	–	
Second line	AZT+3TC+NFV	1 (3.2)	–	
Duration of ART, months	Median (IQR)	16.1 (6.8–30.3)	–	–
Adherence (%)[b]	Good	17 (54.8)	–	–
	Satisfactory	8 (25.8)	–	
	Poor	6 (19.4)	–	

ART, antiretroviral therapy; AZT, zidovudine; CRF, circulating recombinant form; d4T, stavudine; EFV, efavirenz; IQR, interquartile range; NFV, nelfinavir; NVP, nevirapine; 3TC, lamivudine; and URF, unique recombinant form.
[a]HIV serology was determined using New LAV Blot I and II (Bio-Rad Laboratories, Marnes-la-Coquette, France).
[b]Good, 100% pills taken; Satisfactory, ≧95%, but <100% pills taken; Poor, <95% pills taken.
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Table 3. Demographic and clinical characteristics of HIV-1-infected patients <15 years old ($n=11$)[a].

Characteristic		Value
Age, years	Median (IQR)	5.0 (1.5–8.0)
Sex (%)	Female	6 (54.5)
	Male	5 (45.5)
$CD4^+$ T-cell count, cells/μl	Median (IQR)	747 (474–1152)
HIV-1 viral load, log_{10} copies/ml	Median (IQR)	4.3 (3.4–4.8)
HIV-1 genotype (%)	CRF02_AG	7 (63.6)
	URF	4 (36.4)
ART (%)	Naïve	10 (90.9)
	d4T+3TC+EFV[b]	1 (9.1)

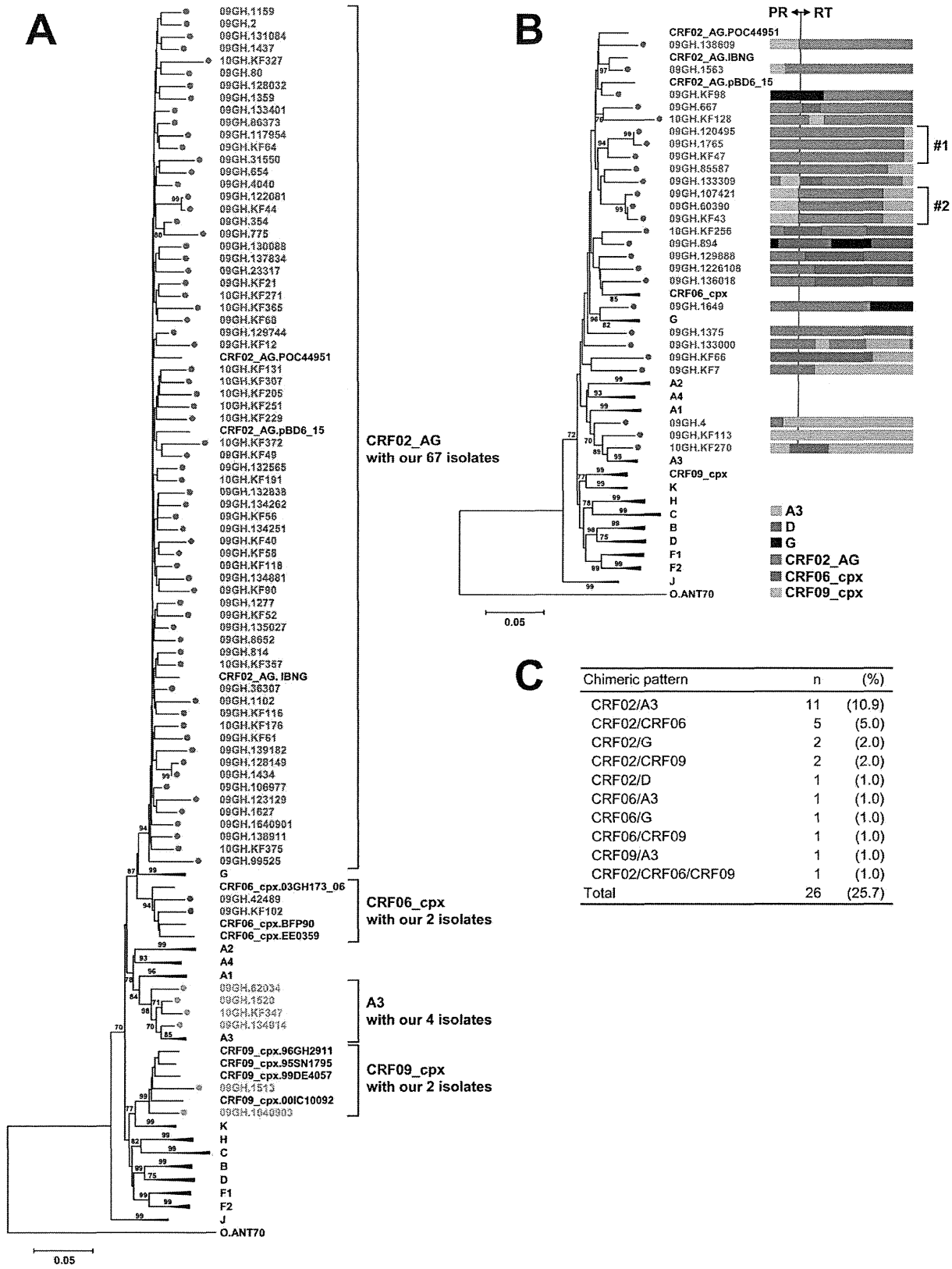
ART, antiretroviral therapy; CRF, circulating recombinant form; d4T, stavudine; EFV, efavirenz; IQR, interquartile range; 3TC, lamivudine; and URF, unique recombinant form.
[a]All were HIV-1 seropositive alone, and their risk factor for infection was mother-to-child transmission.
[b]Only one case had been on treatment for 9.6 months.
doi:10.1371/journal.pone.0071972.t003

NNRTI-resistance mutation loci, V90I, E138A, and V106I, were found in 6 cases (10.2% in Table 4). Our data indicated that drug-resistant HIV-1 transmission events are still low in Koforidua, Ghana.

Eleven children infected with HIV-1 through mother-to-child transmission were also analyzed in our study (Table 3). Their median age was 5.0 years (IQR, 1.5–8.0 years), and 10 of these cases were ART naïve. The remaining case had been treated with d4T+3TC+EFV for 9.6 months but had become viremic. In this case, both 3TC-resistance (M184V) and EFV-resistance (V108I and G190S) mutations were detected (Table 5). Importantly, among the 10 ART-naïve children, a 1.5-year-old case had K103N and G190S NNRTI-resistance mutations (Table 5), suggesting the importance of HIV-1 drug-resistance testing in infants.

Discussion

Our results present a profile of the circulating subtypes and prevalence of drug resistance for HIV-1 infections in Koforidua, Ghana. The data clearly demonstrate the predominance of HIV-1 CRF02_AG (66.3%, $n=67$) in the region (Fig. 1A). Our results, combined with three previous reports on the domination of CRF02_AG in Ghana between 1994 and 2004 [5,6,16], indicate that CRF02_AG has stabilized and maintained its predominance



C

Chimeric pattern	n	(%)
CRF02/A3	11	(10.9)
CRF02/CRF06	5	(5.0)
CRF02/G	2	(2.0)
CRF02/CRF09	2	(2.0)
CRF02/D	1	(1.0)
CRF06/A3	1	(1.0)
CRF06/G	1	(1.0)
CRF06/CRF09	1	(1.0)
CRF09/A3	1	(1.0)
CRF02/CRF06/CRF09	1	(1.0)
Total	26	(25.7)

Figure 1. Molecular epidemiology of HIV-1 infections in Koforidua, Ghana. HIV-1 subtypes of 101 isolates were determined through the construction of phylogenetic trees, similarity plotting, and boot-scanning analyses. (A) Phylogenetic tree containing our 75 isolates classified into known subtypes and CRFs. (B) Phylogenetic tree containing our 26 URF isolates identified with unknown mosaic patterns of the *pol* gene. Two clusters of URF isolates are represented by #1 and #2. (C) Summary on the chimeric patterns of 26 URF isolates. The trees were constructed by the neighbor-joining method. Bootstrap values were calculated from 1,000 analyses, and values greater than 70% are shown at tree nodes. Our isolates are represented by colored circles, and subtype reference isolates are represented by their subtype and name. Scale bar represents nucleotide substitutions per site. HIV-1 group O isolate, ANT70, was used as the outgroup. CRF, circulating recombinant form; PR, protease; RT, reverse transcriptase; and URF, unique recombinant form.
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in the region for nearly 12 years. However, our study identified 26 isolates (25.7%) as URFs (Fig. 1B), indicating that active viral recombinations are ongoing in Ghana. Interestingly, a similar prevalence (25.1%) of HIV-1 URFs was reported from other cities in Ghana, Accra, Agomanya, and Atua [6]. Taken together, these data thus highlight the importance of HIV-1 URFs in understanding the dynamics of the HIV-1 epidemic in Ghana.

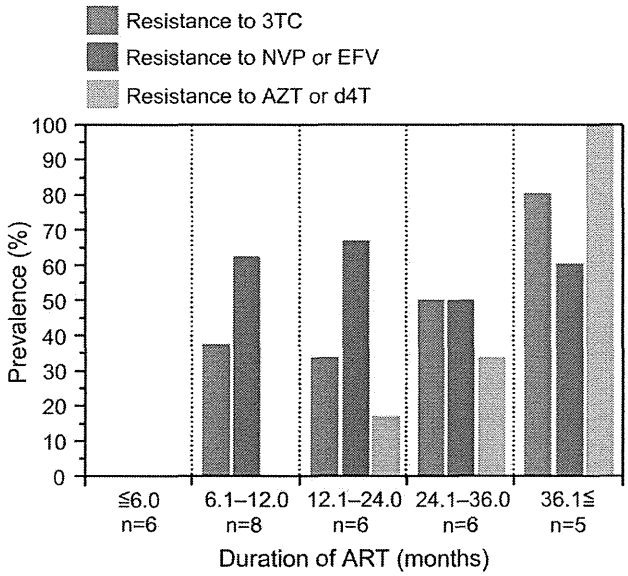
Regarding the situation of HIV-1 drug resistance in Ghana, most of the 31 patients on treatment with virological failure ($n = 22$, 71.0%) had HIV-1 drug-resistance mutations, suggesting

Table 4. Frequency of HIV-1 drug-resistance mutations in ART-experienced and -naïve adult patients (≧15 years old) ($n = 90$)[a].

Mutation	ART-experienced, $n = 31$ (%)	ART-naïve, $n = 59$ (%)
Any	22 (71.0)	6 (10.2)
NNRTI resistance	8 (25.8)	6 (10.2)
NRTI and NNRTI resistance	13 (41.9)	0 (0.0)
NRTI, NNRTI, and PI resistance	1 (3.2)	0 (0.0)
Transmitted drug resistance	Not applicable	0 (0.0)
None	9 (29.0)	53 (89.8)
NRTI-resistance mutation	14 (45.2)	0 (0.0)
A62V	1 (3.2)	0 (0.0)
M184V	12 (38.7)	0 (0.0)
TAMs	8 (25.8)	0 (0.0)
M41L	4 (12.9)	0 (0.0)
D67N	3 (9.7)	0 (0.0)
K70R	4 (12.9)	0 (0.0)
L210W	1 (3.2)	0 (0.0)
T215Y	5 (16.1)	0 (0.0)
T215F	1 (3.2)	0 (0.0)
K219Q	1 (3.2)	0 (0.0)
K219E	1 (3.2)	0 (0.0)
NNTRI-resistance mutation	22 (71.0)	6 (10.2)
V90I	4 (12.9)	4 (6.8)
A98G	5 (16.1)	0 (0.0)
K103N	9 (29.0)	0 (0.0)
V106I	0 (0.0)	1 (1.7)
V106A	1 (3.2)	0 (0.0)
V108I	1 (3.2)	0 (0.0)
E138A	1 (3.2)	2 (3.4)
Y181C	3 (9.7)	0 (0.0)
Y188L	2 (6.5)	0 (0.0)
G190A	2 (6.5)	0 (0.0)
P225H	3 (9.7)	0 (0.0)
M230L	1 (3.2)	0 (0.0)
PI-resistance major mutation	1 (3.2)	0 (0.0)
L90M	1 (3.2)	0 (0.0)

ART, antiretroviral therapy; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; and TAMs, thymidine analog-associated mutations.
[a]HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. For ART-naïve patients, transmitted drug resistance was defined according to the latest definition of the WHO drug-resistance surveillance [11].
doi:10.1371/journal.pone.0071972.t004

A



B

Case	ART regimen	Duration of ART (months)	Amino acid mutations conferring resistance to		
			3TC	NVP or EFV	AZT or d4T
KF375	AZT, 3TC, EFV	6.5		V106A, Y181C	
KF49	AZT, 3TC, NVP	6.8	M184V	K103N, P225H, M230L	
KF43	AZT, 3TC, EFV	8.2		K103N	
9	AZT, 3TC, NVP	8.4	M184V	G190A	
19	AZT, 3TC, EFV	8.9	M184V	K103N	
KF251	AZT, 3TC, NVP	16.1		A98G, K103N	
KF327	d4T, 3TC, EFV	16.3	M184V	K103N, P225H	K70R, K219E
KF347	d4T, 3TC, EFV	17.5		K103N, Y188L	
KF372	AZT, 3TC, EFV	23.3	M184V	K103N, P225H	
KF205	AZT, 3TC, NVP	29.4	M184V	K103N	D67N, K70R, T215Y
15	AZT, 3TC, NVP	30.1	M184V	Y181C	M41L, T215Y
KF229	d4T, 3TC, EFV	34.5	M184V	Y188L	
KF131	AZT, 3TC, NVP	39.9	M184V	A98G, K103N	T215Y
KF102	d4T, 3TC, EFV	41.5			M41L
KF128	AZT, 3TC, NVP	44.6	M184V	V108I, Y181C	D67N, K70R, T215F, K219Q
KF307*	AZT, 3TC, NFV	50.6	M184V	V90I, A98G	M41L, T215Y
KF256	AZT, 3TC, EFV	54.4	M184V	A98G, G190A	M41L, D67N, K70R, L210W, T215Y

Figure 2. Prevalence of 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations by duration of ART in 31 HIV-1-infected patients ≧15 years old. (A) Bar graph and (B) details of 17 patients identified with 3TC-, NVP-, EFV-, AZT-, and d4T-resistance mutations. HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. Amino acid mutations responsible for drug resistance are shown in bold and color coded with bar graph in A. *Major NFV-resistance mutation L90M was found in the protease in the case of KF307. ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; NFV, nelfinavir; NVP, nevirapine; and 3TC, lamivudine.
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that drug-resistant HIV-1 is the major risk factor for virological failure. Furthermore, nearly half of the cases (45.2%, 14/31) had both NRTI- and NNRTI-resistance mutations (Table 4), a pattern that is consistent with that observed in a recent systematic review on treatment-failure cases in sub-Saharan Africa [17], where M184V/I, K103N, and T215Y/F mutations predominate.

Regarding the timing of drug-resistance acquisition, our data demonstrated that 3TC-, NVP-, and EFV-resistance mutations were selected earlier (6.1–12.0 months) than AZT- and d4T-resistance mutations (12.1–24.0 months). Importantly, the prevalence of TAMs increased from 16.7% (1/6) at 12.1–24.0 months to 100% (5/5) at ≧36.1 months. As the accumulation of TAMs confers cross-resistance not only to the first-line NRTIs (AZT, d4T, and 3TC), but also to the second-line NRTIs (ABC, TDF, and ddI) to some extent [18], their accumulation should be avoided by conducting drug-resistance testing earlier and appropriately switching the regimen, once virological failure is suspected.

As no transmitted HIV-1 drug-resistance mutation was found among the 59 newly diagnosed treatment-naïve adult cases, the transmission of drug-resistant HIV-1 appeared to be a rare event in Koforidua, Ghana. Comparing our data with that from other African countries with a similar background, roll-out time of ART, and coverage rate of ART (26.6% in Ghana) [2], the low prevalence of transmitted HIV-1 drug resistance is not surprising and understandable. However, we cannot exclude the possibility of

Table 5. HIV-1 drug-resistance mutations in patients <15 years old ($n = 11$)[a].

ART	n	Amino acid mutations associated with	
		NNRTI resistance	NRTI resistance
Naïve	5	–	–
	2	V90I, V106I	–
	2	V90I	–
	1	**K103N, G190S**	–
d4T+3TC+EFV	1	K101E, V106I, V108I, G190S	M184V

ART, antiretroviral therapy; d4T, stavudine; EFV, efavirenz; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; and 3TC, lamivudine.
[a]HIV-1 drug-resistance mutations were detected according to the latest definition of the International AIDS Society-USA panel [10]. For ART-naïve patients, transmitted drug-resistance (shown in bold and underlined) was detected according to the latest definition of the WHO drug-resistance surveillance [11].
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low levels of transmitted HIV-1 drug resistance in our 59 ART-naïve adult cases. The results of our study are limited by using direct sequencing, which may not have been sensitive enough to detect minority drug-resistant variants hidden among the wild-type strains. Indeed, several studies have reported 2- to 3-fold higher prevalence of drug-resistance transmission with ultra-deep sequencing than with direct sequencing [19,20], which can detect 1% minority populations [21]. Furthermore, as ultra-deep sequencing can better detect the presence of dual or multiple infections of HIV-1 subtypes compared with direct sequencing [22,23], using such new technology may identify subtypes of 26 URFs.

Finally, an eventual increase of transmitted drug-resistance cases is anticipated in Ghana as well. Thus, access to HIV-1 genotypic drug-resistance testing should ideally be expanded along with the scale-up of ART programs. In addition, vertical transmission of drug-resistant HIV-1 was found in one of 10 newly-diagnosed treatment-naïve children, suggesting that expanded access to HIV-1 genotypic drug-resistance testing is also needed for programs to prevent mother-to-child transmission in Ghana.

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Author Contributions

Conceived and designed the experiments: NIN SI JSB KI JAMB SBO SY WKA WS. Performed the experiments: NIN SI JSB KI JAMB. Wrote the paper: NIN SI WS. Organized the study team: KI SY WKA WS. Enrolled patients into the study: SBO. Prepared a clinical database: NIN JSB KI SBO. Revised the manuscript critically JSB KI JAMB SBO SY WKA.

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特集　第60回日本ウイルス学会学術集会
シンポジウム5　海外拠点におけるウイルス感染症研究

8. 東京医科歯科大学がガーナ大学野口医学研究所で展開している2つの感染症研究プロジェクト

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東京医科歯科大学のガーナ大学・野口記念医学研究所共同研究センターは，文部科学省が2005年に開始した「新興・再興感染症拠点形成プログラム」（第Ⅰ期）の後半に追加小規模海外拠点として東北大学・フィリピン拠点と共に採択されたことに基づき2008年に設置されている．同プログラムは2010年4月より第Ⅱ期に入り，名称も「感染症研究国際ネットワーク推進プログラム（J-GRID）」と変え，私たちの共同研究センター（以下ガーナ拠点と呼ぶ）も引き続きその一翼を担うことになって今日に至っている．この野口記念医学研究所で行われている感染症研究は，J-GRIDの他にもう一つあり，それは国際協力事業機構（JICA）と科学技術振興機構（JST）の合同事業である地球規模課題対応国際科学技術協力事業（SATREPS）である．本稿ではこれら2つのプロジェクトを紹介する．

野口記念医学研究所設立の経緯

初めに日本政府の援助で野口記念医学研究所が設立されるまでの歴史的経緯を簡単に紹介する．1957年にBlack Africaとして最初の独立を果たしたガーナは，やがて旧宗主国である英国からの直接的援助が途絶えるようになった結果，同国内の医療機関の設備と技術水準は益々低下する一方であった．この状況を打開すべく，ガーナ政府は1960年代にガーナ医科大学（現ガーナ大学医学部）に対する医療協力の要請を世界各国に対して発出．この協力要請に当初呼応したのが福島県立医科大学であり，この時に陣頭指揮に当たったのが，当時外科教室に赴任して間もない本多憲児教授であった．福島県立医大が手を挙げたのは，遡ること約40年前の1928年に同県会津地方出身の野口英世博士が黄熱病の病原体を探索するために西アフリカのゴールドコースト（現ガーナ）のアクラで不運にも自らが黄熱病の犠牲となり客死したという事実があったことが，その動機の一つであった由である．そしてこれ以後，今日まで一貫して国際協力事業団（JICA）の技術協力プロジェクトなどが続くことになるのであるが，初めの約10年間は主にガーナ医科大学の各教室に対して物的・人的支援が福島県立医大から続けられた．やがて，その方式では供与機材のメンテナンスが悪く，折角導入した先端で高価な研究機器が急速に使用できなくなるなど欠点が散見されるようになり，供与した研究機器を集約した場所に置き研究効率を高めるためにも新しい基礎医学研究所を作ろうという構想が自然に生まれることになった．折から1970年代のオイルショックという非常に困難な日本の財政事情に遭遇したため，すんなりと政府が認可とは行かなかったが，紆余曲折の上，最終的に1979年に完成をみたのが野口記念医学研究所（**図1**，以下「野口研」と省略する）である．この間のいきさつや研究協力内容の詳細については，本多憲児先生が自身の退官記念として刊行された著作物[1]に詳しく述べられている．

野口研の運営方式の歴史的変遷

研究所の本館は一辺の長さが約80mもあるロの字の形をした総2階建の白亜色の建物で，壁などが何度か塗り替えられた他は特に建物の骨格が傷むことなく，ほとんど建築当時のままの姿が現在も保持されているのはなかなか壮観である（**図1**）．そして建物が完成すると同時に，これだけの大規模な研究所への支援を一地方単科大学が続ける

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図1 野口記念医学研究所の外観

ことは至難であるとの判断から，この時を境として福島県立医大は野口研への協力から手を離すことになる．新しい協力方式のスタイルは，複数の大学や国立研究機関等が中核となって国内委員会を形成し，この委員会が主導して人の派遣や研究プロジェクトの中味を決定していくというものであった．こうして1980年代から2000年代半ばまで，ほぼ4年毎に新しい研究プロジェクトのテーマを掲げながら，東京大学医学部を始めとして，国立予防衛生研究所（現国立感染症研究所），名古屋市立大学医学部，三重大学医学部，徳島大学医学部など様々な大学の医学部や関連研究機関から研究者がJICA専門家として派遣されることになった．この過程で東京医科歯科大学もその参加研究機関一つとしてJICA研修員や野口研からの国費留学生の受け入れや専門家の派遣などに深く関わるようになったのが，今日，当大学が野口研をガーナ拠点として直接に担当するようになった経緯である．

野口研で進行している2つの感染症プロジェクト

現在，この野口研では2つの感染症プログラムが並行して進行している．一つは先に述べた国際感染症研究ネットワーク推進プログラム（J-GRID）であり，もう一つはJICAと科学技術振興機構（JST）が共同で行っている地球規模課題対応国際科学技術協力（SATREPS）のプログラムである（**図2**）．丁度2つのプログラム共に2010年4月から5年計画の事業が始まっており（前者は第II期5年間のプロジェクトで，後者は元々5年間計画のプロジェクト），それぞれ4年目に入ったところである．本稿では，前半を井戸が担当しJ-GRIDの研究活動内容について紹介し，後半で山岡がSATREPS事業について紹介することにする．

野口研のJ-GRID研究活動について

野口研におけるJ-GRIDのプログラムでは，現在ウイルス学と寄生虫病学の2分野に各1名の常駐研究者が派遣されている．本稿ではウイルス関連の話題を主な対象としているので，後者の分野に関しては，アフリカ睡眠病の病原体であるアフリカトリパノソーマ原虫の運動機能を司る遺伝子を標的とした新規薬剤候補分子の探索研究や，重要な熱帯感染症の一つであるマラリヤ原虫のガーナ国内における薬剤耐性に関する実態把握研究，更には日和見感染症として特に免疫機能が低下したHIV/AIDS患者に高頻度に現れる疾患であるトキソプラズマ症原虫のゲノムタイピング研究などが行われていることを述べるだけに留めることにする．

ガーナにおける現行ART評価の研究

さて，ウイルス学の分野における研究テーマであるが，次の2つの課題を中心に研究が行われている．

1. ガーナにおける現行のエイズ治療薬（anti-retroviral therapy, ART）の評価研究

★感染症研究国際ネットワーク推進プログラム
Japan Initiative for Global Research Network on Infectious Diseases (J-GRID)

第I期の後半2008年からスタート、2010年より
第II期目に入っている(5年間)　代表　太田伸生

★地球規模課題対応国際科学技術協力
Science and Technology Research Partnership for Sustainable Development (SATREPS)

2010年4月からスタート(5年間)　代表　山岡昇司

図2　野口記念医学研究所で行われている2つの感染症プロジェクトの概要

2. ウイルス性出血熱の研究

ガーナでは2003年に発展途上国向けのエイズ治療薬としてWHO推奨のNRTI　2剤（AZT（もしくはd4T）と3TC）およびNNRTI　1剤（NVPもしくはEFV）の3剤からなる混合薬の処方がARTの第一選択肢として採用されているが(**表1**)，導入から早や10年近くが経過しており，現在どの程度効果があるのかを調べることは緊急の課題なのである．しかしながら，研究設備も機器も十分に備わっていない状況下では，その評価を科学的エビデンスに基づいて正しく下すことは容易ではない．この問題はエイズ治療の前線では最重要課題の一つでもあり，またガーナ側から最も期待される研究テーマでもあった．そこでJ-GRIDの研究テーマとしては，先ずこの現行ARTの有効性評価を取り上げることにした．

方法としては，野口研のある首都アクラから比較的近い距離にある地方病院を定点観測地として，その病院に通院する患者の内，予備的調査によって血中ウイルス量（viral load, VL）やCD4細胞数の測定がなされていて基礎的臨床データが揃っている患者約1,100人をリストアップし，この患者グループを追跡調査の対象とすることにした．毎年，その患者グループの中から一定期間の内に300人分の検体を採取し，VLやCD4の数値を指標としてART成功例グループ(success, S)，ART失敗例グループ(failure, F)，そして部分的成功例グループ（moderately success, M）の3グループに患者を大別することにした．それぞれのグループへの帰属の判定基準に関しては，そのおおまかな指標を**図3**に掲載しているが，要点は薬剤の有効性の判定に関して何よりもウイルス量を制御できているか否かに置いたところにある．従って，VLは抑えられているが，未だCD4の回復が見られていないケースをmoderately success（M）と呼ぶことにし，Sに準ずる扱いとした．この判定基準に関してはおそらく異論のある方も多かろうかと思うが，現行薬剤の継続の可否を評価することに重きを置いたことで斯様なグループ分けを試みたことを理解して頂きたい．2010年から2011年にかけて得られた検体で，それ以前のデータと比較が可能であった234症例に関してグループ分けをした結果を**表2**に示す．全体で見た場合，Sは161 (68.8 %)，Mは43 (18.4 %)，Fが30 (12.8 %) であった．つまり，SとM合わせて約87%では少なくともウイルス量は制御されており，一応現行の処方を続行しても可と判断されるわけである．勿論Mの場合，ウイルス量は抑えられているものの，未だCD4細胞数の回復が見られていない状況なので，今後の病態変化について要観察であることは言うまでもない．約13%がFであったことは，極めて由々しき事態で，これが果たして服薬アドヒアランスの問題であるのか，あるいは既に多剤耐性の変異が患者の体内に感染しているHIV株のポピュレーション全体に拡がっている結果であるのかを明らかにする必要がある．こうした疑問を解明するために患者の血漿中に存在するウイルスゲノムから逆転写酵素遺伝子部分をRT-PCRして配列分析を行うことにより，投与薬剤に対する耐性変異の出現状況の解析などが現在行われている．具体的なデータを示すことはここでは省かせて頂くが，幸いに今のところ多剤耐性変異が一人の患者から得られたHIVのゲノムに出

表1 ガーナで使用されているエイズ治療薬
副作用によってAZTかd4T，またはNVPかEFVの選択は可能であるが，事実上第一選択肢しかない状況と言ってよい．

WHO recommended ART Regimens	
A	First Choice
	Zidovudine + Lamivudine + Nevirapine AZT + 3TC + NVP
	Second Choice
	Zidovudine +Lamivudine + Effavirenz AZT + 3TC +EFV
B	First Choice
	Stavudine+ Lamivudine + Nevirapine d4T + 3TC + NVP
	Second Choice
	Stavudine+ Lamivudine + Effavirenz d4T + 3TC + EFV

現しているケースはほとんど見られないようである．なお**表2**では，最終採血時点においてウイルスが血中に検出された場合と検出されない場合で，これらのグループ分けがどのように変化するかについても示している．（直近の）最終測定時にウイルス量が検出限界以下の数値であった場合，当然のことではあるがSかMに分類され，最終測定時に少しでもウイルスが検出された場合には，それがFである可能性が65%にも上ることが示されている．つまり，このことは，たとえVLの測定は1回だけにしても，そのデータにより治療法の善し悪しを判断することがかなりの確度で可能であることを意味している．

ガーナを含めた西アフリカ一帯の諸国には，世界中に蔓延しているHIV-1の他に，遺伝的・血清学的に異なるHIV-2が地域限定的に存在していることが知られている．このHIV-2はHIV-1に比べて，その感染力や病原性が一般にやや弱いと言われており，事実，最近の統計[2]では，ガーナの全エイズ患者の約94～96%がHIV-1による感染で，HIV-2の感染者は1～2%程度，残りの2～4%はHIV-1とHIV-2の重感染であると報告されている．もしもHIV-2の感染力が本当に弱いのであれば，年々HIV-2の割合は減少する筈であるのであるが，統計を見る限り年度によって増減があるが，必ずしも減少一方でもなく，必ず数%のHIV-2単独感染やHIV-1との重感染があることは注目すべきである．なおHIV-2の感染があった場合，NNRTIはHIVの型特異的にしかウイルス増殖抑制効果がないので，その治療に際しての薬剤の選択には特に注意が必要である．J-GRIDの研究テーマとしては，HIVの流行株に関する分子疫学研究に加えて，こうしたHIV-1とHIV-2が重感染した場合の薬剤効果や病態進行についても着目して現在研究を進めているところである．

野口研におけるウイルス性出血熱研究の状況

ウイルス学として第2の研究テーマであるウイルス性出血熱については，着手してから日が浅く，これまでに予備的調査を終えた段階で，丁度これから具体的成果が見込まれるという状況である．蚊媒介性のウイルス性疾患として黄熱病だけはWHOの監視網に入っていることもあり，それが疑われた患者の検体はガーナ大学医学部付属病院があるコレブ地区内に設置されている公衆衛生実験室（National Reference Laboratory）や野口研などに送付され，黄熱病に関してだけがスクリーニング検査されている．デング熱やウエストナイル熱，さらにはチクングニア熱やリフトバレー熱など他の蚊・ダニなどによる昆虫媒介性ウイルス感染症が存在しているに違いないと推定されるものの，それらの検査体制がほとんどなされていないため，病原体の遺伝子的情報は無論のこと，血清学的情報すら乏しいのが現状である．当然の結果としてそうした昆虫媒介性ウイルス感染症に対するコントロール対策は全く無いに等しいと言

Viral load	CD4		Category
Not detected	Increased	⟹	Success
Not detected	Decreased	⟹	Moderate
Detected (increased VL)	Decreased	⟹	Failure
Detected (decreased VL)	Increased	⟹	Success
	Decreased	⟹	Moderate

図3 ART 評価のために 3 つに分けたカテゴリーの判定基準

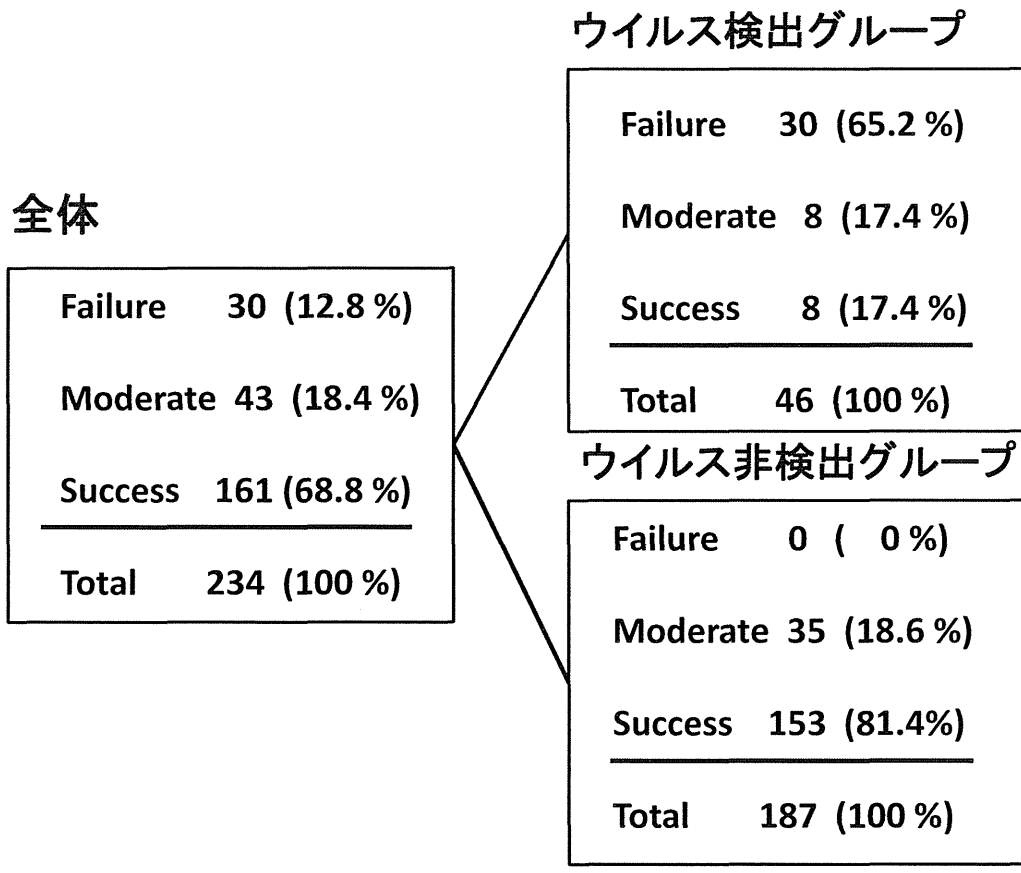

表 2 ART 評価の結果

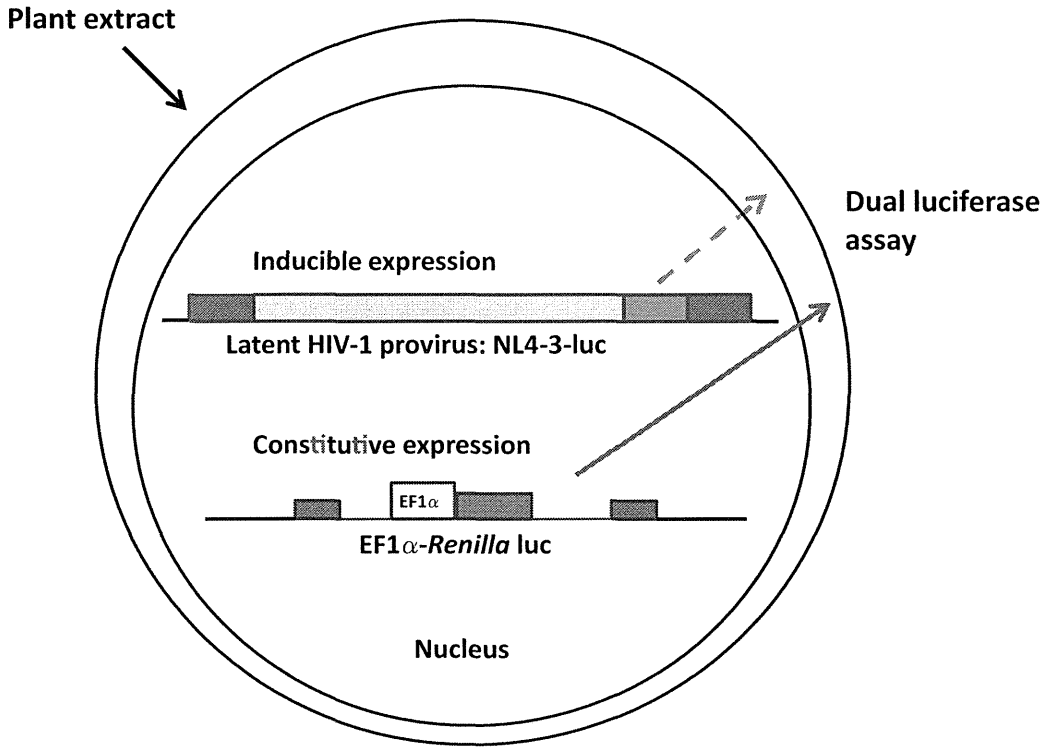

図4 潜伏 HIV-1 プロウイルスの発現を誘導する物質のスクリーニング
潜伏プロウイルス NL4-3luc が組み込まれた Jurkat T 細胞株は，内部標準遺伝子として *Renilla* luciferase 遺伝子を恒常的に発現している．植物抽出物の添加後，両 luciferase 活性を同一サンプルで測定してプロウイルス発現をモニターすることができる．

わざるを得ない．また日本国内では最も危険なⅠ類感染症病原体として指定され恐れられているラッサ熱が西アフリカ諸国一帯には潜在しており，時にアウトブレイクを起こしていることも，同地域の感染症対策を考慮する上で忘れてはならない事実である．今後の野口研におけるウイルス分野における研究活動は，こうしたウイルス性出血熱の領域を重点的に強化しなければならないと考えている．

SATREPS 事業の研究課題「ガーナ由来薬用植物による抗ウイルス及び抗寄生虫活性候補物質の研究」

野口記念医学研究所および生薬科学研究所の要望に基づき，2010 年度から科学技術振興機構（JST）と国際協力機構（JICA）による地球規模課題対応国際科学技術協力（SATREPS）事業として，ウイルス複製，寄生虫増殖を抑制する薬用植物中の抗病原体有効成分の研究を開始した．ガーナでは主に経済的，地理的要因から西洋医学の普及が十分でない一方で生薬に関する伝統的知識は豊富であり，ハーバリストと呼ばれる生薬調合師による伝統医療が全土で普及している．このような現状からガーナ政府は伝統医療のための部局を設置するなどしてその役割を重視しており，本研究に大きな期待を寄せている．ガーナ側では，病原体に関する研究を野口記念医学研究所が，薬用植物の成分抽出は生薬科学研究所が担当している．日本側では，病原体に関しては東京医科歯科大学が，薬用植物の成分抽出と分析については長崎国際大学が担当している．本稿では紙数の都合上，日ガ双方で進められている HIV-1 に関する研究の一端を紹介する．

潜伏感染プロウイルスの発現を誘導する薬用植物由来成分の探索

抗レトロウイルス薬物治療（cART）により血中 HIV-1 RNA 量が減少している場合でも，体内の潜伏感染細胞内にはプロウイルスが残存しており，現行の治療薬では個体からこれを排除することは困難である．ART が中断された際に潜伏感染細胞からウイルスが産生されることがあり，新たな感染と変異ウイルス出現の原因となりうることが知られている．潜伏感染細胞を駆逐するためには，未感染細胞を cART で守りつつ潜伏感染細胞からプロウイルス発現を誘導し，ウイルスの毒性あるいは宿主細胞性免疫によって感染細胞を死滅させる必要がある．Phorbol esters はプロウイルス発現を誘導できる代表的薬剤であるが，しばしば宿主細胞の遺伝子発現も刺激して炎症などの副作用を惹起することが問題である．Histone deacetylase（HDAC）はウイルスの潜伏に重要な役割を果たしている

と考えられ，その阻害剤はex vivo実験で潜伏プロウイルスからの転写を誘導することが報告されているが，感染細胞を死滅させるのにじゅうぶんなウイルス遺伝子発現が得られるか，CD8 T細胞による殺細胞効果が得られることが今後の課題である．本研究では，宿主細胞を不必要に刺激することなく安全にプロウイルス発現を誘導する物質を植物抽出物中に見出すことを目的としている．

2011年度までに，ヒトT細胞株Jurkatをもとに作製したHIV-1潜伏感染細胞株（JLR）を用い，Phorbol 12-myristate 13-acetate（PMA）を陽性コントロールとして植物抽出成分によるプロウイルス活性化のスクリーニングを行ってきた．**図4**に示すように，JLRは内部標準遺伝子産物として*Renilla* luciferaseを恒常的に発現するHIV-潜伏細胞株で，HIV-1プロウイルスに組み込んだfirefly luciferase activityによってプロウイルス遺伝子発現をモニターできる．ガーナ産薬用植物の1次スクリーニングでは，100 μg/mLから3.125 μg/mLまでの濃度に調製した50% EtOH抽出物を試験した．プロウイルス遺伝子発現が刺激により100倍以上誘導されるものを2次スクリーニング候補植物とし，生薬科学研究所でヘキサン，クロロホルム，酢酸エチル，水に分画して活性分画を得ている．このほか，ガーナ産植物の種子から抽出した成分に潜伏プロウイルスの発現を誘導する活性があることを見出し，長崎国際大学薬品資源学正山研究室で有効成分を精製している．この成分は転写因子NF-kappaBをごく一過性に活性化する一方で，ウイルス遺伝子の発現は長時間持続する．NF-kappaBにより発現が制御される遺伝子産物には宿主炎症性サイトカインが多く含まれ，NF-kappaBの持続的な活性化は過剰な炎症の原因となりうる．活性分画による非感染細胞内でのNF-kappaB依存的遺伝子発現を解析するためのレポーター細胞を作成し，潜伏感染モデル細胞としてこのレポーター細胞を抽出物（活性分画）で刺激して発現動態を比較したところ，潜伏感染モデル細胞でのプロウイルス内レポーター遺伝子の発現誘導は持続的であったが，NF-kappaB依存的レポーター遺伝子の発現誘導は一過性であった．ここで持続的なプロウイルスの持続的発現誘導は刺激後初期に発現したHIV-1遺伝子産物TatによるLTRからのmRNA伸長促進の影響を強く受けているためと考えられ，Tatの作用を効果的に活用することの重要性が示唆された．

本事業によってガーナ国内で採取可能な植物材料から感染症対策に有効な物質が見出され科学的エビデンスが付与されれば，今後の持続可能な発展に寄与できるものと期待している．

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Two Research Projects on Infectious Diseases conducted in Noguchi Memorial Institute for Medical Research, University of Ghana by Tokyo Medical and Dental University

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Ghana-Tokyo Medical and Dental University Research Collaboration Center has been established since 2008 when our Program was chosen together with the Program in the Philippines proposed by Tohoku University as an additional small-scale research center of the Overseas Research Program on Emerging and Reemerging Diseases that is funded by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government and started in 2005. This 5-year government-supported Program has changed its name to develop into a more active world-level program called Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and entered the second 5-year phase in 2010, and our Program is playing an important role among other research centers located in Asia and Africa. Currently, two research projects are carried out in parallel in Noguchi Memorial Institute for Medical Research by Tokyo Medical and Dental University: one is a J-GRID project and the other is the one of Science and Technology Research Partnership for Sustainable Development (SATREPS) which is a joint project between Japan International Cooperation Agency (JICA) and Japan Science and Technology Agency (JST). This special article is describing what these two projects are all about.

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Alarin but not its alternative-splicing form, GALP (Galanin-like peptide) has antimicrobial activity

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ABSTRACT

Alarin is an alternative-splicing form of GALP (galanin-like peptide). It shares only 5 conserved amino acids at the N-terminal region with GALP which is involved in a diverse range of normal brain functions. This study seeks to investigate whether alarin has additional functions due to its differences from GALP. Here, we have shown using a radial diffusion assay that alarin but not GALP inhibited the growth of *Escherichia coli* (strain ML-35). The conserved N-terminal region, however, remained essential for the antimicrobial activity of alarin as truncated peptides showed reduced killing effect. Moreover, alarin inhibited the growth of *E. coli* in a similar potency as human cathelicidin LL-37, a well-studied antimicrobial peptide. Electron microscopy further showed that alarin induced bacterial membrane blebbing but unlike LL-37, it did not cause hemolysis of erythrocytes. In addition, alarin is only active against the gram-negative bacteria, *E. coli* but not the gram-positive bacteria, *Staphylococcus aureus*. Thus, these data suggest that alarin has potentials as an antimicrobial and should be considered for the development in human therapeutics.

1. Introduction

In recent years, research in the field of novel antimicrobial peptides has intensified due to the need for newer effective antibiotics to overcome resistance issues of conventional antibiotics. Antimicrobial peptides, isolated from various bacteria, fungi, plants, invertebrates and vertebrates are important components of natural defenses of most living organisms [1]. These molecules have the added advantages of being very small in size, amphipathic and positively charged which allow them to bind and disrupt microbial membranes [2]. Some effective antimicrobial peptides reported thus far include human LL37 which is active against *Staphylococcus aureus* [3,4] and *Escherichia coli* [5] and β-defensins against *Enterococcus faecalis* and *Helicobacter pylori* [6–9].

The galarin family of neuropeptides consists of galanin, a galanin-like peptide (GALP) and a newer member called alarin. Human galanin consists of 30 amino acids and is encoded by the *GAL* gene. GALP consists of 60 amino acids and it can activate galanin receptors (GalRs) because residues 9–21 of GALP are identical to the first 13 amino acids of galanin [10]. Alarin consists of 25 amino acids and is derived from an alternative-splicing of the *GALP* gene that excludes exon 3. Its precursor consists of the signal sequence of the prepro-GALP, the first 5 amino acids of the mature GALP peptide and another 20 amino acids that are not identical to any other peptides. Unlike galanin and GALP, alarin does not bind to GalRs [11,12].

Galanin and GALP mRNAs are widely distributed in CNS as well as in the periphery in GIT, heart, dermis, epidermis, nerves, bone and joint tissues [13]. Alarin mRNA was first detected in ganglionic cells of neuroblastonic tumors [11] and it has a much wider CNS distribution than GALP [14]. It can also be found localized around blood vessels in the skin [11]. Galanin and GALP are involved in a diverse range of normal brain functions such as feeding and

Abbreviations: GALP, galanin-like peptide; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*.

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metabolism, osmotic regulation and water intake, nociception, neuronal injury, survival, regeneration, and neuroprotection, learning and memory and anxiety-related behaviors [13]. Like galanin and GALP, alarin is involved in feeding behavior [12,15,16], food intake and reproductive hormone secretion [12,16,17]. It also has vasoactive and anti-inflammatory activities [15]. The relationship between alarin and GALP is evolutionally and functionally interesting. Alarin shares only 5 conserved amino acids (APAHR) of the N-terminal region with GALP, while all other residues of the C-terminal region are very different. Hence, it is interesting to uncover other novel and specific functions of alarin arising from the differences in amino acid sequence from GALP. This paper investigates the antimicrobial activity of alarin and GALP against *E. coli* and *S. aureus*.

2. Materials and methods

2.1. Materials

Human LL-37, human GALP (galanin-like peptide), and human alarin were purchased from the Peptide Institute, Inc (Japan). Truncated peptides of human alarin [alarin(6–25), alarin(11–25) and alarin(16–25)] were synthesized and purified by IBL co. (Japan). Trypto-Soya Broth was purchased from Nissui (Japan).

2.2. Radial diffusion assay

The antibacterial activities of human LL-37, human GALP (galanin-like peptide), human alarin, and its truncated peptides were all evaluated by radial diffusion assay [18,19], a modification of the sensitive assay for antimicrobial peptides described by Lehrer and colleagues [20,21]. Briefly, to obtain bacteria growth in the mid-logarithmic-phase, an overnight bacterial culture was diluted 1:1000 in Tryptic soy broth (TSB) and was incubated at 37 °C until the optical density of the aliquot reached an absorbance value of 0.4 measured at 620 nm wavelength. The bacteria suspension was centrifuged at 900*g* for 10 min at 4 °C, washed once with ice-cold 10 mM sodium phosphate buffer (SPB; pH 7.4), and was re-suspended in ice-cold SPB. Based on previously prepared standards of the optical density at 620 nm wavelength, a volume containing 1×10^6 bacterial CFU was added to 10 ml of previously autoclaved 10 mM SPB containing 3.0 mg of TSB medium, 1% low-electroendosmosis-type agarose (Sigma), and 0.02% Tween 20.

After rapid dispersion of the bacteria, the agar was poured into an agar plate to form a uniform layer of approximately 2 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 5 μl of antimicrobial agents to each well, the plates were incubated for 3 h at 37 °C and were then overlaid with 10 ml of sterile agar consisting of 6% TSB (double-strength solution) and 1% agarose. Antibacterial activity is identified as a clear zone around the well following incubation for 18–24 h at 37 °C and is measured as the difference in the diameters of the clear zone around the wells containing the antimicrobial peptides and buffer control (3 mm). These experiments were repeated four times.

2.3. Electron microscopy

The effect of LL-37 and alarin on the morphology of *E. coli* was evaluated by scanning electron microscopy. Briefly, the bacteria were treated for 2 h with 20 μM LL-37 or alarin on a MAS coated slide glass (Matsunami co., Japan), air-dried and then fixed with 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). Specimens were further fixed in 1% osmium tetroxide and dehydrated through a graded ethanol series (50%, 70%, 80%, 95%, and 100%) and isopentyl acetate. The dehydrated specimens were then mounted on steel stubs, sputter-coated with a mixture of gold/palladium (Joel JFC-1100), and imaged using a Jeol JSM-840A electron microscope (Jeol, Japan) at 8 kV.

2.4. Hemolysis

Horse blood was purchased from Nihon Bio-Test co. (Japan). Horse blood erythrocytes were rinsed three times in phosphate buffer saline (PBS) by centrifugation at 1000*g* for 10 min and then re-suspended in 8% (vol/vol) PBS. Next, a 50 μl erythrocyte suspension was mixed with 50 μl LL-37, alarin, or GALP and incubated for 1 h at 37 °C. Tween 20 at 2% served as a positive control. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm wavelength and this is expressed as a percentage of the value of Tween 20-induced hemolysis.

2.5. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), with Dunnett's Multiple Comparison Test to identify between-group differences using the GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA), with p values < 0.05 considered as significant. Statistical significance is expressed as $^*p < 0.05$ from data ($n = 4$).

3. Results

The antimicrobial activity of alarin (200 pmol) was compared to its alternative splicing form, GALP and a well-studied antimicrobial peptide human cathelicidin, LL-37. Alarin inhibited the growth of *E. coli* (ML-35), with an increase of ~3.5 mm in the clear zone diameter in comparison to the buffer control (Fig. 1A and B). This inhibitory activity of alarin was also comparable to LL-37 at 200 pmol where its clear zone diameter had an increase of 4 mm when compared to the PBS buffer control (Fig. 1A and B). Its alternative-splicing form, GALP, however, did not show any antimicrobial activity against *E. coli* where the clear zone diameter remained the same as that of the buffer control (Fig. 1A and B). To examine further the dose–response of the antimicrobial activity of alarin; 25, 50, 100 and 200 pmol alarin were used for a radial diffusion assay. Alarin showed significant dose-dependent increases in antimicrobial activity against the gram-negative bacteria, *E. coli* (Fig. 1C). However, a dose up to 200 pmol alarin did not show any antimicrobial activity against the gram-positive bacteria, *S. aureus* (Fig. 1D). LL-37 in micromolar concentrations have been reported to induce bacterial membrane blebbing, leading to a leaky membrane and cell death in *Burkholderia pseudomallei* and *Burkholderia thailandensis* [22,23]. Using electron microscopy, we showed that 20 μM LL-37 induced extensive membrane blebbing on *E. coli* (Fig. 2B) compared to the buffer control (Fig. 2A). Membrane blebbing was also observed on *E. coli* incubated with alarin at 20 μM but is less extensive than those caused by LL-37 (Fig. 2C).

To explore the role of the N-terminal region of alarin, antimicrobial activity of truncated peptides of alarin, alarin (6–25), alarin (11–25), and alarin (16–25) were examined by a radial diffusion assay (Fig. 3). Alarin (6–25) and alarin (11–25) showed weak antimicrobial activity, while the antimicrobial activity of alarin (16–25) was completely abolished (Fig. 3). These results suggest that both the C-terminal and N-terminal regions of alarin are essential for its strongest killing effect.

It was reported that LL-37 has hemolytic activity on erythrocytes [22]. To determine the hemolytic activity of alarin and GALP, 10 μM of each peptide were incubated with horse erythrocytes for

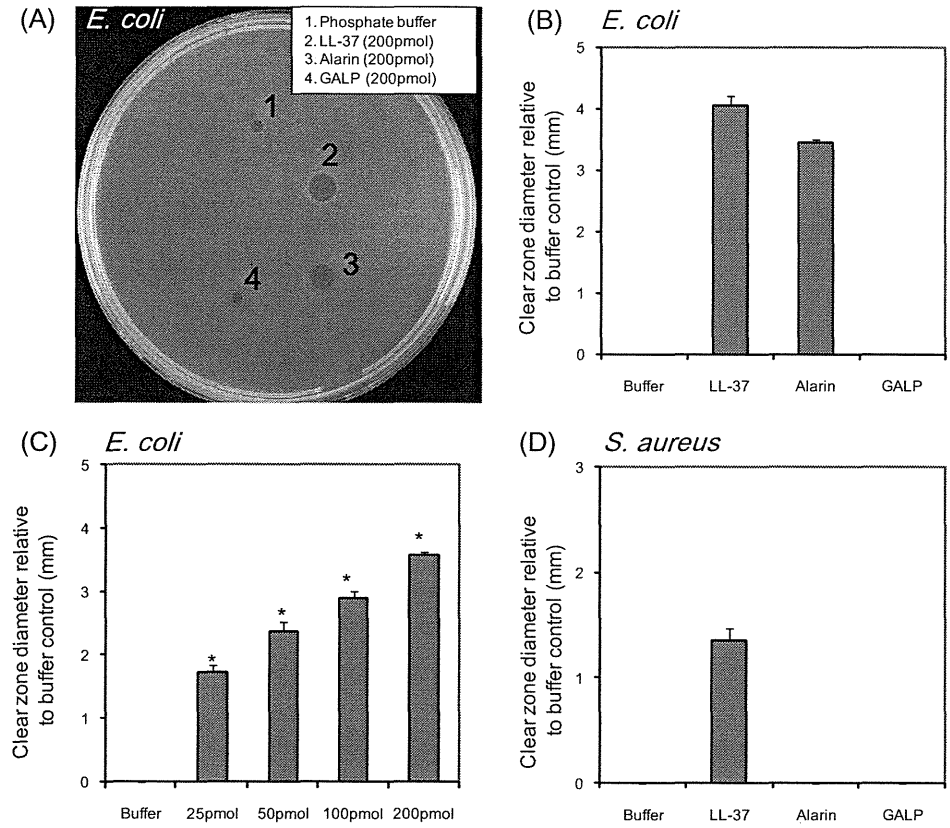

Fig. 1. Antimicrobial activities of LL-37, alarin and GALP against *E. coli* ATCC43827 (ML-35) and *S. aureus* ATCC25923 by radial diffusion assay. (A) Bacterial culture plate used for radial diffusion assay of LL-37, alarin and GALP. (B) Clear zone diameter (mm) depicting antimicrobial activity of LL-37, alarin and GALP at 200 pmol. (C) Dose-dependent antimicrobial activities of alarin against *E. coli* ATCC43827 (ML-35) and (D) dose-dependent antimicrobial activities of alarin against *S. aureus* ATCC25923. Zone diameter (mm) represents the antimicrobial activity of various concentrations of alarin relative to buffer control. An increase in the zone size caused by alarin is obtained by subtracting the diameter of the buffer control well (3 mm). Statistical significance is expressed as $^{*}p < 0.05$ (one-way ANOVA with Dunnett's Multiple Comparison Test) versus buffer control. Each bar represents the mean ± SD of data ($n = 4$).

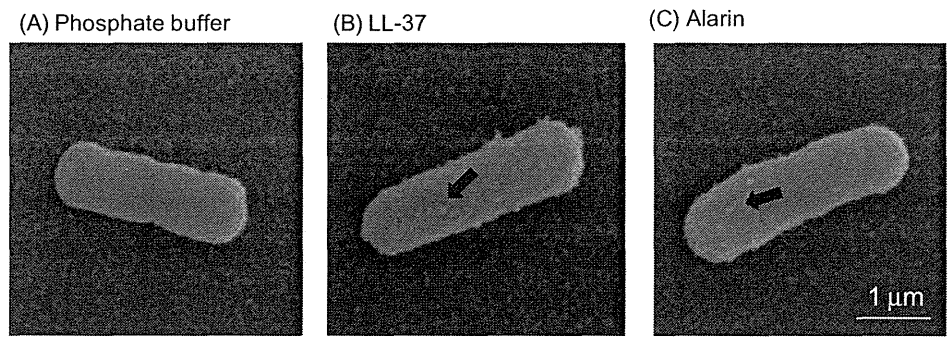

Fig. 2. Electron micrographs of *E. coli* ATCC43827 (ML-35) cells. (A) *E. coli* incubated with phosphate buffer showed normal surface of membrane; bacteria incubated with (B) 20 μM LL-37 and (C) 20 μM alarin showed blebs (black arrows) on the membranes. Scale bar, 1 μM.

1 h at 37 °C. LL-37 showed hemolytic activity to horse erythrocytes but not to alarin and GALP (Fig. 4).

4. Discussion

Alarin is differentiated from its alternative splicing form, galanin-like peptide (GALP) at the C-terminal region. Although alarin shares some similar functions with GALP, it is unknown if alarin has other more specific roles. Antimicrobial peptides are characterized as amphiphilic molecules and consist of basic amino acids. Because the C-terminal region of alarin contains some basic amino acids such as Arg and Lys, we postulated that alarin may have antimicrobial activity.

Results from the present study showed that alarin has antimicrobial activity but unlike the human cathelicidin, LL-37 which has a broader spectrum of antimicrobial activity, the antimicrobial activity of alarin is limited to the gram-negative bacteria, *E. coli*. This is not surprising as different susceptibilities of bacterial species against a particular peptide have been observed and this can be attributed to various reasons such as bacterial cell surface

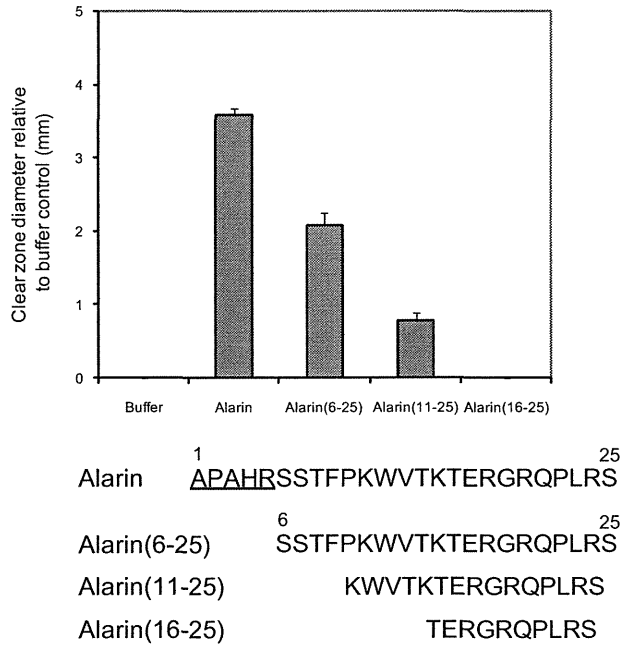

Fig. 3. Antimicrobial activities of alarin and its truncated peptides against *E. coli* ATCC43827 (ML-35) by radial diffusion assay. Clear zone diameter (mm) depicting antimicrobial activity of alarin and its truncated peptides at 200 pmol (upper panel) and amino acid sequences of the truncated peptides (lower panel). Conserved sequences of alarin and GALP are underlined.

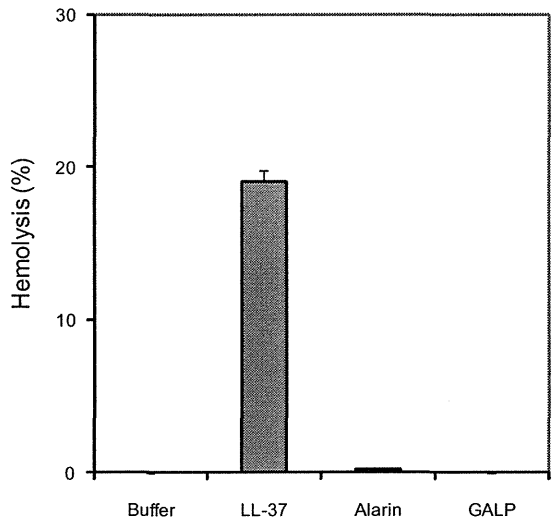

Fig. 4. Hemolytic activity of LL-37, alarin and GALP. Horse erythrocytes were incubated with each peptide (10 μM) for 1 h at 37 °C. Hemolytic activity is a measurement of the percentage of Tween 20-induced hemolysis. LL-37 showed hemolytic activity against horse erythrocytes but not alarin and GALP.

charge [24], genetic diversity [25], host ionic conditions [26] and peptide-to-lipid ratios [27]. The antimicrobial activity of alarin, however, is specific as alarin exhibited dose-dependent inhibition of the growth of *E. coli* and demonstrated potency equal to that of LL-37. We further showed by electron microscopy that alarin is capable of causing bacterial membrane blebbing, suggesting that membrane disruption could be one of its killing mechanisms. An effective antimicrobial peptide should be cytolytic and cell-penetrating but not hemolytic [28]. In general, hydrophobic interactions with eukaryotic cell membranes increase hemolytic activity [29]. Our results showed that alarin does not induce hemolysis of erythrocytes, suggesting that its hydrophobic interactions with the erythrocyte membranes are minimal. Hence, alarin can fulfill the desirable features of an effective antimicrobial peptide.

More interestingly, GALP, an alternative splicing form of alarin demonstrated no antimicrobial activity. However, deletion of the conserved amino acid sequence (APAHR) at the N-terminal region of alarin reduced its antimicrobial activity suggesting that APAHR remain essential for its antimicrobial activity. The C-terminal original sequence of alarin (6–25), which contains some basic amino acids are important for the antimicrobial activity of alarin as successive deletions reduced and eventually abrogated the antimicrobial activity of alarin. These results, hence, suggest that even though the C-terminal region of alarin confers its antimicrobial activity, the conserved N-terminal of alarin and GALP remains crucial for its maximum antimicrobial activity.

LL-37, defensins and histatins are some of the well-studied antimicrobial peptides which contain 37, 29–42 and ~32 amino acids, respectively [30]. With the discovery of its antimicrobial activity, alarin with a length of 25 amino acids is now one of the shortest antimicrobial peptides known. Results from this report show that alarin has antimicrobial potentials and should be considered for the development as a human therapeutic.

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HEALTH AND DEMOGRAPHIC SURVEILLANCE SYSTEM PROFILE

Profile: The Mbita Health and Demographic Surveillance System

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The Mbita Health and Demographic Surveillance System (Mbita HDSS), located on the shores of Lake Victoria in Kenya, was established in 2006. The main objective of the HDSS is to provide a platform for population-based research on relationships between diseases and socio-economic and environmental factors, and for the evaluation of disease control interventions.

The Mbita HDSS had a population of approximately 54 014 inhabitants from 11 576 households in June 2013. Regular data are collected using personal digital assistants (PDAs) every 3 months, which includes births, pregnancies, migration events and deaths. Coordinates are taken using geographical positioning system (GPS) units to map all dwelling units during data collection. Cause of death is inferred from verbal autopsy questionnaires. In addition, other health-related data such as vaccination status, socio-economic status, water sources, acute illness and bed net distribution are collected.

The HDSS has also provided a platform for conducting various other research activities such as entomology studies, research on neglected tropical diseases, and environmental health projects which have benefited the organization as well as the HDSS community residents. Data collected are shared with the community members, health officials, local administration and other relevant organizations. Opportunities for collaboration and data sharing with the wider research community are available and those interested should contact shimadam@nagasaki-u.ac.jp or mhmdkarama@yahoo.com.

Why was the HDSS set up?

The mission of the Institute of Tropical Medicine at Nagasaki University (also known as Nekken in abridged Japanese or NUITM in Kenya) is to overcome tropical diseases, particularly infectious diseases, and the various health problems associated with them, in cooperation with related institutions, and to strive for excellence in the following areas:

(i) spear-heading research in tropical medicine and international health;
(ii) global contribution through disease control and health promotion in the tropics by applying the fruits of the research;