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Ebola virus circulation in Africa: a balance between clinical expression and epidemiological silence.

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Résumé : Circulation du virus Ebola en Afrique : équilibre entre expression clinique et silence épidémiologique.

Environ trente ans après les premières épidémies, on connaît encore peu de choses sur le virus Ebola (VEBO), sa transmission reste indéterminée et son réservoir difficile à identifier. Peu de temps après l'épidémie de fièvre Ebola et la découverte du virus en 1976, plusieurs pays, y compris des zones écologiques, firent l'objet d'une étude approfondie au début des années 80 afin d'étudier la répartition du VEBO en Afrique centrale : la République de Centrafrique (RCA), le Cameroun, le Tchad, le Congo, le Gabon et la Guinée-Equatoriale. Depuis 1992 le test ELISA ainsi que la RT-PCR ont été utilisés pour la recherche d'anticorps du virus spécifique et pour la caractérisation de l'ARN virale. Les zones d'épidémies, géographiquement très éloignées les unes des autres, laissent supposer que le réservoir et le cycle de transmission du VEBO sont sans doute étroitement liés à l'écosystème de la forêt tropicale, hypothèse confortée par la répartition des anticorps. Les épidémies, peu fréquentes, semblent indiquer la présence d'un réservoir animal rare, ou écologiquement isolé ayant peu de contact avec l'homme et les primates non-humains. Cependant, des recherches sérologiques diverses ont montré une forte prévalence chez l'homme sans signalement de pathologie. Cela indiquerait une circulation des souches pathogènes, non pathogènes, et plus de contacts avec l'homme que prévus ; cela pourrait également expliquer, en partie, le silence des quinze années de fièvre Ebola entre émergence et ré-émergence du virus, dans le bassin congolais. Aujourd'hui, largement éclairée par l'étude des récentes manifestations épidémiques et épizootiques du VEBO au Gabon et pays avoisinants, l'histoire naturelle du VEBO commence à être comprise pour ce qui est des principes de l'épizootie chez les primates non-humains ainsi que la chaîne de transmission.

Summary:

Nearly thirty years after the first epidemics, Ebola virus (EBOV) remains hardly described, its transmission unclear and its reservoir elusive. Soon after the Ebola fever outbreak and virus discovery in 1976 and in order to investigate the distribution of EBOV in Central Africa, several countries including a range of ecological zones were investigated in the early 1980s, using extensive survey: Central African Republic (CAR), Cameroon, Chad, Congo, Gabon and Equatorial Guinea. Since 1992, ELISA antibody test along with a RT-PCR have been used to detect specific virus antibodies and characterize viral RNA. The widely separated geographic locations of outbreaks have suggested that the reservoir and the transmission cycle of EBOV are probably closely associated with the rain forest ecosystem, what is supported by the distribution of antibodies. The fact that outbreaks seldom occur suggests the presence of a rare or ecologically isolated animal reservoir having few contacts with humans and non-human primates. However various serological investigations showed a high prevalence in humans without any pathology reported. This suggests a circulation of both pathogenic and non-pathogenic strains as well as more frequent contacts with man than expected, and could partially explain fifteen years of Ebola fever silence between the emergence and re-emergence of Ebola virus in the Congolese basin. Nowadays, largely enlightened by the study of recent epizootic and epidemic manifestations of EBOV in Gabon and neighboring countries, EBOV natural history starts to be understood as for the fundamentals of epizootic in non-human primates and chains of transmission.

virus Ebola
Filovirus
fièvre hémorragique
primate
Afrique centrale

Ebola virus
Filovirus
haemorrhagic fever
primate
Central Africa

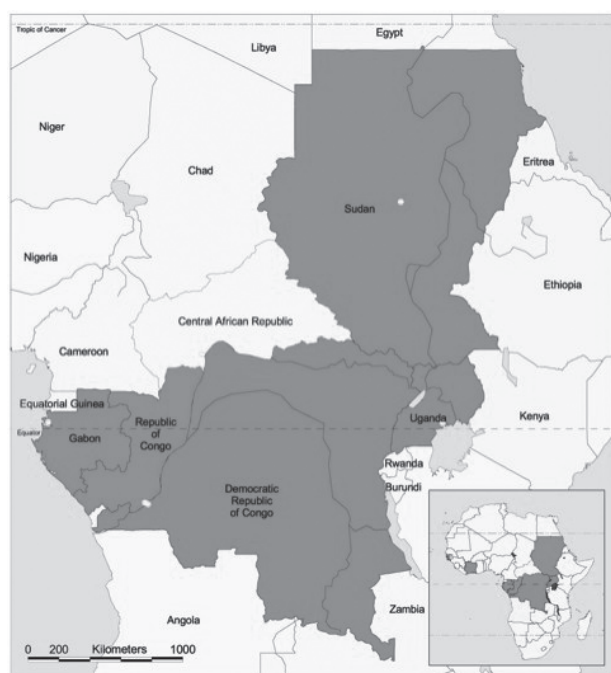
Introduction

Ebola virus (EBOV) infection, causing severe hemorrhagic diseases in human and non-human primates (39, 23), was identified for the first time as a deadly epidemic at Nzara in Southern Sudan (45) and at Yambuku in Northern Democratic Republic of Congo (DRC, former Zaire) in 1976 (20). Since then, EBOV epidemics have been reported in several other Central African countries, Gabon, RC and Uganda (figure 1), during two distinct periods with a fifteen years gap of silence. EBOV are non-segmented negative-strand RNA viruses, classified in the family Filoviridae, together with Marburg virus. Genetic and antigenic characteristics of the Ebola viral strains isolated allowed to identify four different types, named after their geographical origin: Zaire Ebolavirus (EBOV-Z), Sudan Ebolavirus (EBOV-S), Côte-d'Ivoire Ebolavirus (EBOV-CI) and Reston Ebolavirus (EBOV-R). The high mortality rate, from 50 to 90%, the absence of vaccine or treatment and the ignorance of the mechanisms of its sporadic emergence make EBOV infections one of the most dangerous diseases for humans. Most of the cases resulted from inter-human transmission (45, 20) and remained not informative about its natural reservoir, potential vector, and mode of transmission.

Figure 1.

Central African countries affected by Ebola outbreaks.

Pays d'Afrique centrale affectés par les épidémies dues au virus Ebola.



Since the first epidemics, research programs have been conducted all over Central Africa, in both epidemic (cited previously) and non-epidemic countries (Senegal, Guinea, Liberia, Côte-d'Ivoire, Burkina Faso, Benin, Nigeria, Cameroon, Equatorial Guinea, Central African Republic (CAR), Chad, Ethiopia, Kenya, Zimbabwe, Botswana, Madagascar and the Philippines), to solve the mysterious unknown of EBOV natural transmission and maintenance cycle. These studies focused on tracking EBOV scars and identifying vulnerable populations and environmental factors of EBOV emergence, in assumed endemic areas throughout the tropical rain forest. Domestic and wild animals were sampled to find potential hosts, reservoirs or vectors of virus. The scope of studies, the evolving

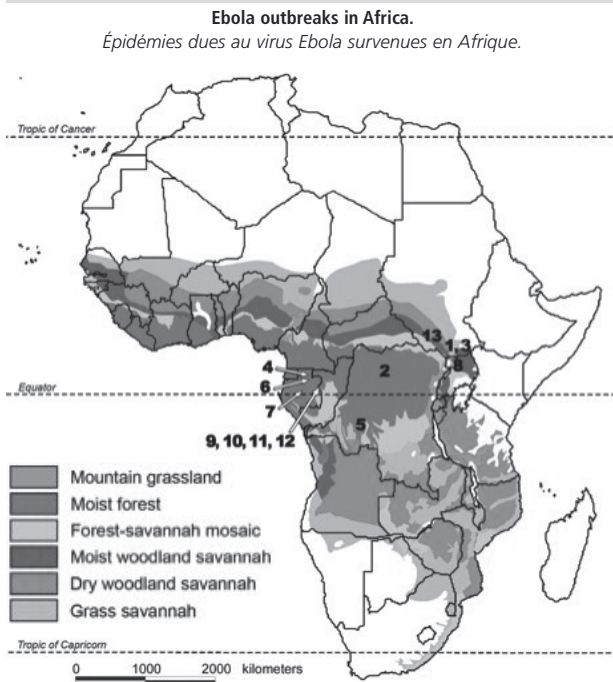
techniques used as well as the unsuspected results make of particular interest the review of more than two decades of investigation. Herein, we report this extensive prevalence and ecological survey, first in epidemic countries exploring the epidemiological silence, then beyond the known places of emergence, with the aim of identifying vulnerable populations, and lastly investigating the highly diverse fauna to understand its wild transmission cycle.

Ebola virus in epidemic Central African countries: regular infections behind temporal coincidences of outbreaks

Two periods of coincident outbreaks fifteen years apart

In 1976, EBOV was first identified in association with two consecutive outbreaks from June to November in southern Sudan and in September and October in northeastern DRC, causing respectively 284 cases with 53% mortality (45) and 318 cases with 88% mortality (20). Nzara, the village and cotton factory where the first Sudanese Ebola outbreak occurred, lies 150 km from the Central African Republic (CAR) border, in the savannah-forest mosaic domain that covers one third of CAR. Less than 150 km south of the Oubangui River, which is the natural border between Northern DRC and CAR, are the villages of Yambuku and Tandala, in the boucle of Ebola River, where first Zairian Ebola infections occurred. Both villages are part of the large phytogeographical domain of the Congolese rain forest, which extensively covers the southern part of CAR. DRC was hit again in 1977 by an isolated case and southern Sudan in 1979 by a fourth outbreak, causing 34 cases among five families, with 65% mortality (2). After a long silence of more than 15 years, Ebola epidemics occurred repeatedly starting in 1994 in two different sub-Saharan African countries: Gabon in 1994 (52 cases with 60% mortality) and during two periods in 1996 (January to April: 31 cases with 68% mortality; and July to January 1997: 59 cases with 75% mortality) (10, 11, 54) and DRC in 1995 (315 cases with 81% mortality) (23). In 1994 an ethologist was also infected during the autopsy of a wild chimpanzee in Côte-d'Ivoire (25). In the 1996-1997 outbreak, a case of Ebola hemorrhagic fever was diagnosed in a nurse for the first time in South Africa. The patient got infected while being exposed to the blood of an ill doctor, who was contaminated in Libreville, Gabon. In 2000-2001, Uganda recorded the largest epidemic, causing 425 cases (mortality rate = 53%) (53). Then in 2001 in Gabon, Ogooué Ivindo province, where occurred the three consecutive epidemics from 1994 to 1997, was affected by a fourth epidemic (65 cases with 82% mortality) linked to a following outbreak in the neighboring Republic of Congo (RC), at Mbomo and Kéllé (59 cases with 75% mortality), (29-31). Later, two epidemics reoccurred in RC, in the same area: in 2002-2003 at Yembelen-goye and Mvoula, with subsequent spread respectively at Kéllé and Mbomo (143 cases with 89% mortality); and in 2003-2004 at Mbandza with subsequent spread respectively at Mbomo (35 cases with 83% mortality). The last epidemic was recorded in May 2004 in Yambio county, southern Sudan, and caused 17 cases with 41% mortality. To date 13 Ebola epidemics and two isolated cases have been recorded, causing a total of 1848 cases and 1288 deaths, according to the WHO (54); all of them occurred in tropical Africa (figure 2). Cases have never been reported in the Philippines from where EBOV-R is originated. Identified in October 1989, in Reston (Virginia, USA), in infec-

Figure 2.



- 1- 1976: Nzara-Maridi, Sudan
- 2- 1976: Yambuku, Democratic Republic of Congo (DRC, ex-Zaïre)
- 3- 1979: Nzara, Sudan
- 4- 1994-1995: Minkébe-Mekouka, Gabon
- 5- 1995: Kikwit, DRC
- 6- 1996: Mayibout, Gabon 7- 1996-19
- 7: Bououé, Gabon
- 8- 2000-2001: Gulu-Masindi, Uganda 9- 2001-2002: Ekata, Gabon
- 10- 2001-2002: Mékambo-Mbomo-Kellé, Republic of Congo (RC)
- 11- 2002-2003: Kellé-Mbomo, RC
- 12- 2003: Mbomo-Mbandza, RC 13- 2004: Yambio, Sudan

ted cynomolgous monkeys from the Philippines, EBOV-R has only been associated with epidemics in monkeys (*Macaca fascicularis*) and asymptomatic illness in humans, while other EBOV subtypes induce hemorrhagic fever in both humans and monkeys, with various degrees of pathogenicity (34).

Even if some sporadic cases were probably not observed, all of Ebola epidemics have shown both temporal and spatial coincidences:

- They emerged during two distinct periods: 3 between 1976 and 1979 and 10 between 1994 and 2004. No clinical case was declared between these two periods.
- Spatially they all occurred in a tropical African ecosystem, located between the latitudes of 5° north and 5° south.

Serological watch following epidemics

Starting in 1979, several research programs on *Filovirus* epidemiology have been conducted in Central Africa, including extensive serological survey and clinical awareness in assumed endemic areas. More than 15,000 sera and biological samples have been tested for EBOV to identify endemic areas and vulnerable populations (table I). Most of the studies performed the indirect immunofluorescent antibody test (IFA), described by WULFF and LANGE (55). IFA was adjusted for EBOV by JOHNSON *et al.* (21), and, since then, has been largely used in serological surveys to estimate viral antigens. The

lack of specificity of IFA tests should be mentioned, regarding the high seropositivity recorded. However IFA tests could prove seropositivity in the epidemic areas, first under surveillance: DRC (15, 17), Sudan (2), Gabon (16, 10), Uganda (41) and RC (12). Extended surveys then revealed that EBOV infections still occur while no cases are reported inside as well as outside the known distribution of former epidemics. During the long silence following the first outbreak series, surveillance showed periodic infections without epidemics. Between 1981 and 1985, JEZEK *et al.* investigated the region of Tandala in DRC, where the 1977 single case was identified. Suspect cases were reported by local Public Health facilities and tested for EBOV infection by IFA (titer $\geq 1:64$) (17). Among them, 18 persons from 14 different localities were seropositive, which represented a 1% seropositivity related to 137 villagers selected as a control. Following the 1995 outbreak in Kikwit, DRC, BUSICO *et al.* investigated different populations using ELISA test for the detection of EBOV-Z IgG antibodies (8). They found 2.2% prevalence among workers (9/414) living in and around the city and a greater prevalence, 9.3% (15/161) among villagers in areas not affected by the 1995 outbreak, showing higher vulnerability in closer proximity to the forests. These findings raised the complexity of EBOV outbreak, defying all logic in regard to the high case-fatality rate and recurrent infections without

Table I.

Prevalence of EBOV infections from human serological data in Africa and Asia, since 1961.
prévalence d'infections du virus Ebola à partir de données sérologiques en Afrique et en Asie depuis 1961.

year	country	total tested	% Ebola prevalence	test*	references
1961-62	Ethiopia	277	19.8	IFA (>1:16) Z	TIGNOR <i>et al.</i> , 1993
1977	DRC ¹	984	4	IFA (>1:64)	VAN DER GROEN <i>et al.</i> , 1978
1977	Sudan (Maridi)	214	33	IFA	VAN DER GROEN <i>et al.</i> , 1978
1977	Sudan (Nzara)	218	6.4	IFA	VAN DER GROEN <i>et al.</i> , 1978
1977	Rhodesia	243	3	IFA	VAN DER GROEN <i>et al.</i> , 1978
1977	Panama	200	2	IFA	VAN DER GROEN <i>et al.</i> , 1978
1978	DRC	1096	7	IFA (>1:16)	HEYMANN <i>et al.</i> , 1980
1978	Liberia	481	6	IFA	KNOBLOCH <i>et al.</i> , 1982
1979	CAR ²	1344	3	IFA	SALUZZO <i>et al.</i> , 1982
1979	Sudan	23	25	IFA (>1:16)?	BARON <i>et al.</i> , 1983
1979-82	CAR	1909	4.5	IFA	GONZALEZ <i>et al.</i> , 1983
1980	Kenya	84	5	IFA	SMITH <i>et al.</i> , 1982
1980	Gabon	253	7.5	IFA (>1:16) S/Z	IVANOFF <i>et al.</i> , 1982
1980	Zimbabwe	486	2	IFA (>1:16)?	BLACKBURN <i>et al.</i> , 1982
1980	Cameroon	1517	9.7	IFA (>1:16)?	BOUREE <i>et al.</i> , 1983
1981	Zaire	138	5	IFA	STANFIELD <i>et al.</i> , 1982
1981	Kenya	741	1.4	IFA (>1:16)	JOHNSON <i>et al.</i> , 1982
1981-82	Liberia	225	13	IFA (>1:16) Z	VAN DER WAALS <i>et al.</i> , 1986
1981-85	DRC	137	1	IFA (>1:64) S/Z	JEZEK <i>et al.</i> , 1999
1982-83	Guinea	138	8	IFA (>1:16) Z	BOIRO <i>et al.</i> , 1987
1983	Ethiopia	250	0	IFA (>1:16) Z	TIGNOR <i>et al.</i> , 1993
1983	Burkina Faso	992	0	IFA (>1:64) S/Z	GONZALEZ unpubl., 1986
1983	Benin	603	0.3	IFA (>1:64) S/Z	GONZALEZ unpubl., 1986
1984	CAR	296	2.6	IFA (>1:64)	MEUNIER <i>et al.</i> , 1987
1984	Kenya	471	10	IFA (>1:16) S/Z	JOHNSON <i>et al.</i> , 1986
1984	Uganda	132	3	IFA (>1:16) S/Z	RHODAIN <i>et al.</i> , 1989
1984	Senegal	650	22.9	IFA (>1:64)	SALUZZO unpubl., 1987
1984-86	Botswana	154	0	IFA (>1:16) S/Z	TESSIER <i>et al.</i> , 1987
1985	Gabon	213	9.4	IFA (>1:64)	MEUNIER <i>et al.</i> , 1987
1985	CAR	659	22	IFA (>1:64)	MEUNIER <i>et al.</i> , 1987
1985	Cameroon	375	2	IFA (>1:16)?	PAIX <i>et al.</i> , 1988
1986-87	Nigeria	1677	2	IFA (>1:16) S/Z	TOMORI <i>et al.</i> , 1989
1987	CAR	4295	21	IFA (>1:16) S/Z	JOHNSON <i>et al.</i> , 1993
1987	CAR	427	17	IFA (>1:128) S/Z	JOHNSON <i>et al.</i> , 1993
1987	Chad	334	3.6	IFA (>1:16) S/Z	GONZALEZ <i>et al.</i> , 1989
1987	Cameroon	1152	7.7	IFA (>1:16) S/Z	GONZALEZ <i>et al.</i> , 1989
1987	RC ³	728	10	IFA (>1:16) S/Z	GONZALEZ <i>et al.</i> , 1989
1987	Equatorial Guinea	688	16.1	IFA (>1:16) S/Z	GONZALEZ <i>et al.</i> , 1989
1988	Zimbabwe	486	1.9		BLACKBURN <i>et al.</i> , 1992
1988	Madagascar	381	4	IFA (>1:16) S/Z	MATHIOT <i>et al.</i> , 1989
1989-90	Philippines	186	6	IFA (>1:128) Z/R	MIRANDA <i>et al.</i> , 1991
1995	CAR	190	13.2	ELISA Z	GONZALEZ <i>et al.</i> , 2000
1996	Gabon	205	23	ELISA S/Z/R	GEORGES <i>et al.</i> , 1999
1996	Philippines	231	0.4	ELISA	MIRANDA <i>et al.</i> , 1999

1. DRC : Democratic Republic of Congo (ex-Zaïre), 2. CAR : Central African Republic, 3. RC : Republic of Congo

* Z: EBOV-Z, S: EBOV-S, CI: EBOV-CI, R: EBOV-R

diseases, suggesting a circulation of both pathogenic and non-pathogenic strains.

Learning from primates Ebola infections

Populations of non-human primates have been particularly affected by EBOV epidemics. During a two-year epidemic in the Taï National Forest (Côte-d'Ivoire) half of the population of chimpanzees disappeared (25). Simultaneous epizootic have been observed in non-human primates in the area of human outbreaks, as for the discovery of at least 64 died gorillas, chimpanzees and duikers before and during the 2001 human Ebola outbreaks in Gabon, in spite of the rapid decomposition of carcasses in the tropical forest (31). The number of animals, asymptomatic hosts of the virus, in the Ogooué Ivindo region is probably much higher. Although non-human primates have been a source of EBOV infection for humans, they are not designated as a reservoir. The study of the mechanisms of their infection could inform on those leading to the transmission of EBOV from a natural reservoir to humans. Between 1985 and 2000, LEROY *et al.* conducted a first serological survey of EBOV in 790 non-human primates, belonging to 20 species, in Cameroon, Gabon and RC (31). A 12.9% seroprevalence (ELISA) of EBOV-Z IgG antibodies in wild-born chimpanzees attest a continuous contact of chimpanzees with EBOV. The results support the idea of a wide circulation of EBOV and, furthermore, of filoviruses in a large ecotone, the Central African rain forests, starting long before the first human outbreaks. Localized in a narrow equatorial belt, rain forests are characterized by dense vegetation on several strata with an inner colder climate. Interactions between species, either plants or animals, within rain forests can be interpreted as a symbiotic ecosystem, for which human activity (forest opening, logging, agriculture or settlement) are particularly harmful. These ecological changes account for the pressure on fauna forcing the population into new environment and behavior (movement of rain forest fauna, introduction of savannah species) and could partially explain multiple contacts between host and virus reservoir or vector. Likewise the ecotone forest-savannah in the Congolese domain or savannah in Sudan is also an area of EBOV epidemics extending the range of EBOV distribution. Furthermore the EBOV 12.9% seroprevalence rate found in wild-born chimpanzees indicates that, like humans, they can recover from EBOV infection or can be infected but remain asymptomatic (28, 31). Thus, beyond some eventual cases not reported or wrongly diagnosed, the long epidemiological silence of Ebola outbreak does not necessarily preclude its absence.

Ebola virus scars without reported cases: a persistent threat in the Congolese rain forest

Considering the ecological context of Ebola epidemics in Sudan and Zaire in 1976, CAR was chosen in 1979 as country site regarding both cultural and environmental similarities. In a collaborative effort between the Institut Pasteur, Bangui, the Centers for disease control, the United States army medical research institute for infectious diseases, at Fort Detrick and, the Organisation de coordination pour la lutte contre les grandes endémies en Afrique Centrale, at Yaoundé (12, 13, 18, 19), a large serological surveillance was conducted from 1979 to 1982 to determine the frequency and distribution of seroprevalence against several hemorrhagic fevers of viral origin. A preliminary study in eastern

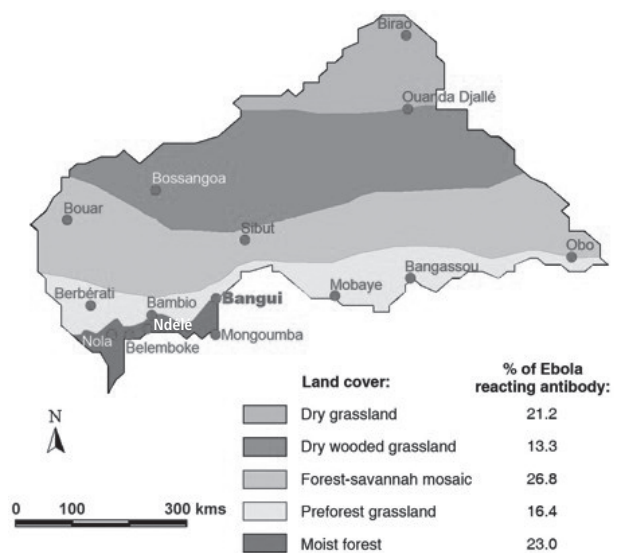
Central African Republic tested 499 human sera samples by IFA and reported 3.4% (17 cases) positive with EBOV-Z or EBOV-S antigen and a high antibody titer ($\geq 1:64$) with 3 sera, revealing serological scars of potential past contacts with EBOV (42). From a total of 1909 human sera tested by IFA ($\geq 1:64$) in CAR, 4.5% were positive with EBOV-Z or EBOV-S (12). ELISA techniques, recommended later for their higher sensibility and better specificity, were not available for the diagnosis of EBOV infections at this time. In order to decrease the specificity of IFA, only sera with a high antibody titer ($\geq 1:64$ for human and animal sera) were ultimately considered as potentially positive for Ebola virus reacting antibodies and titrated in an attempt to delineate the EBOV endemic area in Central Africa.

From 1985 to 1987, 5,070 randomly selected human sera from 6 Central African countries (Chad, Cameroon, Central African Republic, Equatorial Guinea, Gabon and Republic of Congo) were tested by IFA (antibody titer $\geq 1:64$) for EBOV, in the

Figure 3.

Seroprevalence of Ebola virus between 1992 and 1999, by ecological zone, in Central African Republic (CAR).

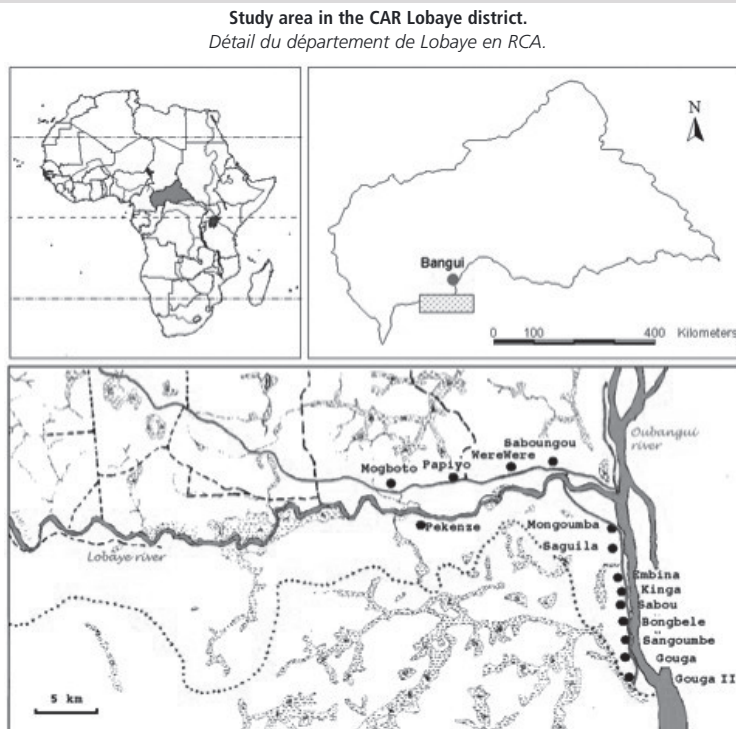
Séroprévalence du virus Ebola en République Centrafricaine (RCA) entre 1992 et 1999, par zone écologique.



frame of a serosurveillance of hemorrhagic fevers (12). Both rural and urban areas were investigated, over different ecotones from dry savannah to tropical rain forest. 12.40% of sera were positive to EBOV-Z or EBOV-S antibodies.

From 1992 to 1995, a deep EBOV-Z antibody serological investigation was carried out in Lobaye district, south-west of Bangui, where previous EBOV antibody high seroprevalence was detected (13, 18). Lobaye district is covered by both preforest grassland and Congolese rain forest (figures 3 and 4). The study pygmy camps and non-pygmy villages are located in a remote forested area, some along the west bank of the Oubangui river, and the others along the north bank of the Lobaye river, its major tributary. Three groups of pygmy camps (Sangoumbe, Sakoungbou and Mogboto) and village group (Gouga villages data combined in results and discussion) were investigated. Pygmies who participated in the study belong to the Baka group (or Aka) (figure 4). Despite increased contact with the villagers living in the forest, most of the Pygmies from that area have preserved a characteristic semi-nomadic lifestyle based on hunting and gathering of spontaneous natural resources in the rain forest. Villagers from Gouga

Figure 4.



belong mainly to Mbuti tribe (Bantu locutors) and, the others to the Mbanza and Ngbundu tribes (Banda locutors). These people practice subsistence farming, hunting, limited trapping and fishing activities. Sera were collected in November 1995 at the beginning of the dry season. The results of EBOV-Z antibody seroprevalence analysis were compared with tests on sera sampled in other provinces, sharing similar environments: Belemboke in December 1992 and November 1994, Nola in December 1995 and Bangassou in October 1996 (figure 3). A direct ELISA test for immunoglobulin G (IgG) detection, as described by KSIAZEK (24), was realized at the Institut Pasteur of Bangui. Each serum, prepared in solutions at four different dilutions (14) was tested against EBOV-Z antigen and control antigen. Sera were considered as positive if their titer was greater than or equal to 1:400 and the sum of the optical density of all four dilutions was greater than 1.000. EBOV-Z reacting antibodies were detected in each village and camp investigated in the Lobaye district (table II), as well as in the compared areas, Belemboke, Nola and Bangassou (table III): 5.3% of the population investigated was seropositive for EBOV-Z IgG antibodies. Although the sample sizes were not always sufficient to be statistically significant, some trends were still observed. The Pygmy Aka population consistently showed higher antibody prevalence against EBOV-Z (13.2%) than the non-pygmy villagers (4%). Pygmies living in Belemboke presented an increasing EBOV-Z antibody prevalence between 1992 and 1994 ($p=0.003$) without clinical manifestation. The same sera also tested negatively for IgM antibodies. In the non-Pygmy population living in Belemboke, Nola and Bangassou, Seroprevalence in villagers living in spoilt forests in Nola and Bangassou was close to those recorded at Kikwit between two epidemics, in a similar biotope. Among Pygmy populations, seroprevalence was recorded higher in females (15.3%) than in males (10.9%) but not statistically significant (χ^2 , $p=0.15$), while it was significantly higher among the 21-30 age group ($p<0.05$). Activities, such as cooking or hunting, associated with the preparation of meat in forests, are risk factors increasing the exposition to the disease. Another survey

conducted in 1997 in Lobaye district showed a non-significant increase of the seroprevalence ($p=0.12$) of the villagers and employees of a logging company, compared with the state in 1995. A higher seroprevalence was noticed for the employees working in forests than those working in the logging company (unpublished data). These investigations concluded on an active circulation of EBOV without any diagnosed cases (wrong diagnoses of Ebola as other fevers are not excluded) and the potential association between infections and human behavior. However, contacts between humans, monkeys and the potential reservoir remain unknown.

Ultimately, considering the fifteen years of absence of epidemics, the apparent extensive distribution and high prevalence of EBOV reacting-IFA in human sera from Central Africa (including CAR, Gabon, Cameroon, Equatorial Guinea, Chad and Republic of Congo) and the lack of specificity of the IFA test, despite its high sensitivity, new techniques were used to test for a second time in 1996, sera previously collected before the 1990s and tested at the Institut Pasteur and USAMRIID by IFA a decade ago. Respectively 185 and 296 human sera collected in 1984 and 1987 within CAR rain forest were tested again at the Institut für Virology at Marburg using an ELISA Test (EBOV-Z antigen) and two other tests

of confirmation, WB and/or IFAT. No significant difference ($p>0.01$) of EBOV-Z antibody prevalence was found between the IFA test performed a decade ago (6.4%) and the tests done in 1996 (6.2%). Sera, positive first and negative the second time, showed a decrease of antibody while the ones negative first and positive later can be the consequence of the cut off of >128 or the antigenicity of the strains used in the upgraded test. Such findings confirmed the presence of EBOV antibodies in CAR human populations ten years ago suggesting that Lobaye populations have been in contact with an EBOV or an EBOV-like antigen since that time. Every serum was also tested by ELISA against the EBOV-R antigen (district from the African strain and from a Philippine origin) and was negative. EBOV-infected patients showed a rapid decrease of IFA antibodies 30 months after onset, while ELISA antibodies remained at

Table II.

EBOV antibody prevalence (IgG, ELISA $\geq 1:400$), by ethnic group and sex in Lobaye District, Central African Republic, 1995.

Prévalence d'anticorps du virus Ebola (IgG, ELISA $\geq 1:400$), selon l'ethnie et le sexe dans le Lobaye District, République de Cente Afrique, 1995.

village	ethnic group	male*	female*	total*
Gouga	Bantus	0/26 (0)	2/24 (8.33)	2/50 (4.0)
Sangoumbé	Pygmy	2/23 (8.7)	4/28 (14.3)	6/51 (11.8)
Sakoungbou	Pygmy	4/24 (16.7)	4/24 (16.7)	8/48 (16.7)
Mogboto	Pygmy	4/45 (8.9)	7/46 (15.2)	11/91 (12.1)
total		10/118 (8.5)	17/122 (13.9)	27/240 (11.2)

* positive/total tested (%)

Table III.

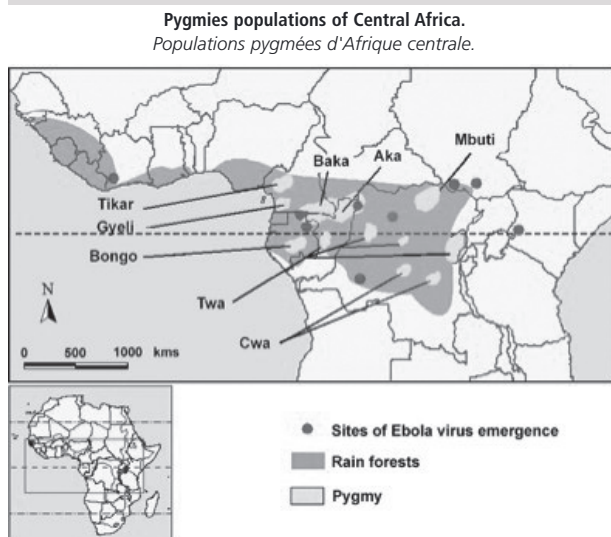
EBOV antibody prevalence (IgG, ELISA $\geq 1:400$) in other investigated areas.

Prévalence d'anticorps du VEBO (IgG, ELISA $\geq 1:400$) dans les autres régions d'enquête.

location	Pygmies*	villagers*
Belemboke CAR, 1992	7/361 (1.9)	-
Belemboke CAR, 1994	16/132 (12.1)	3/99 (3.1)
Bangassou CAR, 1995	-	8/226 (3.6)
Nola CAR, 1995	-	10/278 (3.6)
Lobaye CAR (Batalimo, 1997)	-	21/205 (10.2)
Gordil CAR, 1998	-	0/29 (0)
Kikwit RDC, 1990	-	5/181 (2.8)
Ngoila Cameroon, 1998	-	0/21 (0)

* positive/total tested (%)

Figure 5.



a relatively high titer (Bernard LE GUENNO, pers. comm.). Because all of the positive sera, in the present study, reacted by both ELISA and IFA tests, it is reasonable to infer that some human populations of CAR rain forest were exposed for a long period of time to an active EBOV circulation.

Results provide some geographic indications about the potential location of the chain of transmission, occurring either in epidemic or non-epidemic countries. EBOV circulation in the Congolese rain forest constitutes a considerable threat on local populations, strengthening the need of an effective early warning system to detect precursor signs of potential outbreak, a quest for which the knowledge of the reservoir and vector is a key.

Ebola virus natural transmission: an elusive reservoir and vector

Following the first epidemics, animals in closest contact to humans were trapped around the outbreak areas and unsuccessfully tested for the identification of any potential reservoir species. In Yambuku area (DRC), JOHNSON KM inoculated 818 bedbugs, 15 mosquitoes, 123 rodents, eight squirrels, seven bats, ten domestic pigs, six monkeys, two duikers and one cow into Vero cell cultures, without succeeding in recovering EBOV (20). At the beginning of 1977, in Nzara province, ARATA and JOHNSON B. also failed in identifying EBOV from 501 specimens of the most common mammalian species, including in particular 309 rodents and 178 bats (1). In 1979, two years after EBOV-Z infected an isolated case at Tandala, in DRC, BREMAN *et al.* collected in DRC and Cameroon 1664 animals, including 463 bats, 137 squirrels, 514 other rodents and 267 primates (7). They were tested, one more time unsuccessfully, by IFA, and Vero cells cultures for antibody detection and virus isolation, recognizing the lack of specificity of serological tests.

A permanent «watch» effectively started in 1979 at the edges of the CAR forest, involving a multidisciplinary approach of the biodiversity of small terrestrial mammals (13). Among 1331 animals from different species, tested for EBOV antibody seroprevalence by IFA ($\geq 1:64$), 17.3% of dogs, 16.3% of domesticated guinea pigs and 4.5 to 7.7% of *Mastomys* were positive with EBOV-Z or EBOV-S (table IV). These findings were not conclusive by that time regarding the IFA lack of specificity.

In 1995, following the Kikwit epidemic in DRC, LEIRS *et al.* tested unsuccessfully 3066 vertebrates, including 1914 rodents, for EBOV-Z IgG antibodies by ELISA and virus isolation (26). A similar investigation was conducted by REITER *et al.* on 27,843 arthropods, most of which were 15118 mosquitoes and 6538 bedbugs (40). All ELISA tests, as well as attempts of viral isolation, were negative.

In 1996-1997, FORMENTY *et al.* could find EBOV-CI antibodies, using ELISA in a monkey species, *Colobus badius*, among 1650 vertebrates collected in Taï forest (9). Unfortunately EBOV-CI could not be isolated.

In 1999 and 2000, MORVAN *et al.* tested 223 animals (162 rodents, 24 shrews, 32 birds and 5 primates) for IgM and IgG EBOV antibodies by ELISA. All the specimens were negative to EBOV IgM antibody. Only one rodent, *Praomys* sp. (from 41 trapped), and one shrew, *Sylvisorex ollula* (10 trapped), were positive for IgG antibodies. Then molecular techniques (RT-PCR) were used on 947 animals to detect EBOV glycoprotein or polymerase gene sequences in RNA extracts of the organs (37). Trapped between 1994 and 1999, these animals include essentially small mammals: 98%, but also 16 birds and 2 reptiles. Seven specimens belonging to two genera of rodents (Muridae: *Mus setulosus*, *Praomys* sp1 and *Praomys* sp2) and one species of shrew (Soricidae: *Sylvisorex ollula*) were positive. Neither live virus nor virus antigen was detected in any organ sample. However RNA and DNA were extracted from positive organs and RNA sequences encoding fragments of EBOV glycoprotein were amplified (36). Direct sequencing of amplicons identified the virus as being of the EBOV-Z subtype. Virus-like nucleocapsids were observed by electron microscopy in the cytoplasm of the spleen cells of one animal. These species are common in the study area and share ecosystems bordering forests. These results suggested that a common small mammal living in peripheral forest areas has been in contact with EBOV and attested the persistence of EBOV RNA and DNA in the organs of animals.

Conclusion

Since the disease recognition and the Ebola virus isolated and characterized, serological scars have been observed, beyond the places of known outbreaks, throughout Cen-

Table IV.

Animals collected in Central African Republic from 1979 to 1983 and tested for EBOV antibody prevalence (IFA $\geq 1:128$).

animaux recueillis en République de Centre Afrique de 1979 à 1983 et ayant subi un dépistage d'anticorps du virus Ebola (IFA $\geq 1:128$).

	habitat*	total tested	% EBOV positive (IFA)
shrew	F	5	0
rodents		709	
<i>Arvicanthis</i> spp.	S	98	9.2
<i>Mastomys</i> spp.	F	91	7.7
<i>Mastomys</i> spp.	S	265	4.5
<i>Mus</i> spp.	F	54	1.8
<i>Praomys</i> spp.	F	195	0
<i>Praomys</i> spp.	S	2	0
<i>Taterillus</i> spp.	S	4	0
other mammals		617	
cattle	F	98	0
cattle	S	20	10.0
chicken	S	131	10.0
dog	F	133	17.3
dog	S	29	34.5
donkey	S	13	23.1
goat	F	21	0
goat	S	75	0
pig	F	80	16.3
sheep	S	17	0
Total		1331	

*F = forest, S = savannah

tral Africa. Previous observations and studies suggested that unknown *Filovirus* with variable pathogenicity may exist in different parts of the World (3). EBOV strain distribution in Africa appears limited to a distinct ecosystem geographically associated with the rain forest belt. Epidemics seem to be related to the behavior of human and non-human primates more than a spontaneous circulation of a highly pathogenic strain. The Ebola strain from the Taï forest of Côte-d'Ivoire has been found in the same ecological zone as its most closely genetically-related Ebola-Zaire strain (44). Such divergence between the two strains and their association within the same forested domain can account for their common origin, since the Ivorian forested massif was at one time part of the Congolese rain forest and became separated during the last glaciations (16,000 years before present). By analyzing sequences of Marburg and Ebola viruses, SUZUKI and GOJOBORI estimated the divergence time between the two viruses to be more than several thousands years ago (47). Molecular analysis is and will be the key for clarifying the remaining Ebola mysteries.

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