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Table of Content: Volume 9 Number 22, 3 June, 2015

ARTICLES

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Review

Chiropteran and Filoviruses in Africa: Unveiling an ancient history

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***Ebolavirus* and *Marburgvirus* belong to the Filovirus family and are responsible for hemorrhagic fevers in Africa. The first documented Filovirus outbreak in Africa occurred in Central Africa and was attributed to *Ebolavirus* species. In the last four decades, Filoviral hemorrhagic fevers (FHFs) outbreaks caused by Ebola and Marburg viruses have been on the increase in Africa. The 2013-2015 outbreak has been the largest outbreak in human and has had the most devastating human and economic impact. Epidemics usually originate from a primary single introduction of the virus into simian or human population followed by an interspecies spill over. Multiple, short and isolated transmissions to humans have been also observed. Since the 1976 Yambuko (Democratic Republic of Congo) and Nzara (Sudan) epidemics, several investigations of different animal species have been undertaken but failed to identify the natural reservoirs of *Ebolavirus*. Further studies identified bats as probable reservoirs of *Ebolavirus* in Gabon, and major natural reservoirs of *Marburgvirus* in Uganda, supposed central forested areas of Africa as the epicenter where these viruses originated from, before dissemination. Chimpanzees, gorillas and duikers have been identified as highly sensitive hosts of *Ebolavirus* within wildlife. However, the relative importance of potential vertebrate hosts in the FHFs emergence into human population remains unclear. Different transmission routes involving bats have been proposed. Filoviruses have a zoonotic origin; amplified and maintained in nature between potential reservoirs in a jungle cycle. *Ebolavirus* mostly escapes these natural foci, when other sensitive secondary simian are infected and transmit the virus to human population via hunting, bat's saliva infected wild fruit collection or land monitoring, while *Marburgvirus* emergence was linked to monkey's tissues handling or human entry into bat sheltering habitats. This review discusses the dissemination of filoviruses circulating within their possible chiropteran reservoir species. Vertebrate hosts suspected in the maintenance/transmission cycles are reviewed and their bioecological features discussed. Despite the importance of the findings about reservoirs' discovery, several other questions such as plurispecific associations, migration routes, breeding cycles need to be addressed and are pointed out in this review, in order to generate risk maps for filoviruses' (re)emergence in West Africa.**

Key words: *Ebolavirus*, *Marburgvirus*, Chiropteran, emergence, bioecology, West Africa.

INTRODUCTION

Filoviral hemorrhagic fevers (FHFs) are endemic to Africa. Certainly confined in a jungle cycle for a long time, their etiological agents, namely Ebola and Marburg viruses circulated silently without any manifestation in human population until 1976, when *Ebolavirus* hemorrhagic fever was first simultaneously diagnosed from human communities in Yambuko (Democratic Republic of Congo, DRC) (Johnson, 1978) and Nzara and Maridi (Sudan) (Smith, 1978). Its closest relative, *Marburgvirus* was first recognized in Marburg, Germany and Belgrade, Serbia (formerly Yugoslavia) in 1967 causing an outbreak of severe viral hemorrhagic fever among laboratory workers. African green monkeys (*Chlorocebus aethiops*) imported from Uganda for research purpose were the source of the infection (Smith et al., 1967; Siegert et al., 1968). In Africa, it appeared first in Johannesburg, South Africa (Gear et al., 1975). Since those first recorded emergences, filoviruses increasingly manifest their pathogenic potential, sporadically emerging or re-emerging, enlarging their areas of incidence into Africa and threatening public health and animal biodiversity. There has been a mystery overlapping their natural emergence for decades. Nowadays, bats are much more known involved in their transmission cycle. The emergence of *Ebolavirus* in West Africa inspired several interrogations and request detailed research-action studies in order to understand the extent that the viral amplification, within the reservoir species, has reached. It is likely that the 2013 Guekedou emergence in Guinea was induced by a fruit bat, *Eidolon helvum* (Funk and Piot, 2014). If the virus circulates within the local West African fauna, it will then have the opportunity to set in new ecological niches, in a West African sylvatic cycle, and sporadic epidemics are predictable in West Africa. Surveillance study programs across West African countries, along a west-east prospection transect bordering the northern limit of the forested areas of Central Africa needs to be entirely undertaken. This will aim to detect virus circulation or specific antibodies in reservoir and incidental hosts using serology and RT-PCR for viral nucleic acid sequences detection from wild samples in order to infer the natural history of *Ebolavirus* circulation, and map the geographic range of the virus' amplification. This review discusses the filoviruses associated with bats, and proposes future directions for epidemiological and ecological studies that need to be undertaken, in order to better understand the involvement of chiropteran populations and the patterns of FHFs emergence.

We reviewed the literature on chiropteran found naturally infected with filoviruses in Africa. Other bat species or wild animals from which filovirus nucleic acid sequences or serological evidence of filovirus circulation has been detected are also listed. Considering the ecological and ethological features so far known about chiropteran (Rosevear, 1965; Walker, 1999), we speculate on the potential filoviruses' extension due to their migration, roosting and reproduction.

A literature analysis allowed us to discuss each potential reservoir species' implication in the epidemiology of Ebola and Marburg viruses. Future orientation studies are proposed to pinpoint the areas at risk for eventual filovirus' emergence in West Africa. Systematic terminology of chiropteran used in this paper follows Rosevear (1965) and Walker (1999), while classification of filoviruses follows the revised filovirus taxonomy of the 9th report of the International Committee on Taxonomy of Viruses (ICTV) (Kuhn et al., 2010; 2013). The distribution maps of bats are documented from the available bibliographic data and unpublished collection data from the IRD laboratory of medical zoology, in Dakar, Senegal. We hypothesize the potential amplifying mechanisms, and the ways from which human populations might become infected from sylvatic cycles. We also specify the eventual role of various potential bat reservoir species.

BACKGROUND OF FILOVIRAL HEMORRHAGIC FEVER OUTBREAKS

Filoviruses, the causative agents

The causative agents of FHF are non-segmented, enveloped negative-sense, single-stranded RNA viruses, that morphologically resemble rhabdoviruses and functionality paramyxoviruses, similar also in their genome organization, expression and replication (Feldmann et al., 1993; Beer and Kurth, 1999). RNA viruses have a high ability to rapidly evolve in response to changing host and environmental circumstances via multiple genetic mechanisms, what classify them among the most dangerous emerging and re-emerging pathogens (Morens and Fauci, 2013). The family *Filoviridae* (filo derived from: *filum*, Latin) comprises three genera: *Ebolavirus*, *Marburgvirus* and *Cuevavirus*. The two first ones are the most known because they were described during deadly filoviral hemorrhagic fever epidemics. A third genus, *Cuevavirus*, (species *Lloviu cuevavirus*) less known than the precedents, was only described after a filoviral outbreak [viral pneumonia due to Lloviu virus (LLOV)] which affected a population of the Schreiber's bats, *Miniopterus schreibersii* Kuhl, 1817 in Spain, Europe (Negredo et al., 2011). The genus *Ebolavirus* includes

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five genetic and antigenic subtypes: *Bundibugyo ebolavirus* (BEBOV), *Zaire ebolavirus* (ZEBOV), *Reston ebolavirus* (REBOV), *Sudan ebolavirus* (SEBOV) and *Taï Forest ebolavirus* (TAFEBOV) or *Ivory Coast ebolavirus* (ICEBOV). The genus *Marburgvirus* accounts for a single species, *Marburgvirus marburgvirus* (formerly *Lake Victoria marburgvirus*), which consists of two very divergent "viruses": Marburg virus and Ravn virus, approximately 20% divergent at a genetic level (Carroll et al., 2013; Kuhn et al., 2010, 2013; Towner et al., 2006, 2009). This is in contrast to the known diversity for Ebolavirus species, with *Zaire ebolavirus* having only a 2.7% nucleotide difference between sequences, *Sudan ebolavirus* 5.2%, and *Reston ebolavirus* 4.5% (Lauber and Gorbalenya, 2012; Carroll et al., 2013).

Despite increasing numbers of viruses being detected, some species are represented by single viral lineage (for example, *Taï Forest ebolavirus* by Forest virus and *Lloviu cuevavirus* by Lloviu virus). During the 1998 Marburg Viral Disease outbreak that occurred in northeastern DRC, nine genetic lineages of the virus were involved (Bausch et al., 2006). In 1976, when *Ebolavirus* described 9 years after *Marburgvirus* presented the same filament-like structure as *Marburgvirus*, both were included in the same family of *Filoviridae,* newly described (Kiley et al., 1982). With the growing awareness of the rising threats to humans and wildlife caused by filoviruses, the importance of bats as potential reservoirs of viruses are much more investigated and will probably provide more divergent lineages within *Filoviridae*, that will enrich these taxonomic classifications.

Discovery of filoviruses

Ebolavirus

The first emergences of *Ebolavirus* were documented from Yambuko (DRC), Nzara and Maridi (Sudan) in 1976 with very high case fatality rates of 88 and 53%, respectively, caused by two distinct species of *Ebolavirus*: *Z. ebolavirus* (ZEBOV) (Johnson, 1978), and *Sudan ebolavirus* (SEBOV) (Smith, 1978). The source of transmission remains unknown. The causative agent was then named *Ebolavirus* after the Ebola River running along the Yambuku village, in the North Equator province of the Democratic Republic of Congo (formerly Zaire), where it was first diagnosed in the human population in 1976, simultaneously as in Nzara, Sudan (Smith, 1978). The number of cases has risen steeply and *Ebolavirus* outbreaks re-emerged after a long silent period (1980-1993), with increased frequency and new species discovery: *Côte d'Ivoire ebolavirus* (CIEBOV) in 1994 in the Ivory Coast and, *Bundibugyo ebolavirus* (BEBOV) in 2007 in Uganda (Towner et al., 2008). While re-emerging in Gabon and Republic of the Congo, *Ebolavirus* incidence in human was concomitant with a marked mortality amongst gorillas and chimpanzees infected with the ZEBOV strain. *Ebolavirus* epidemics occurred between latitudes 10°N and 10°S, on both sides of the equator (Peterson et al., 2004; Groseth et al., 2007), approximately corresponding to the Afrotropics, with exception of *S. ebolavirus* which emerged at the extreme Eastern. The disease spread from Central to West Africa. Four of the known *Ebolavirus* species have emerged in sub-Saharan Africa, causing deadly outbreaks: *S. ebolavirus* (SEBOV), *Ivory Coast ebolavirus* (CIEBOV), *Bundibugyo ebolavirus* (BEBOV), and *Zaire ebolavirus* (ZEBOV) recently incriminated in the biggest Ebola epidemic ever recorded touching Guinea, Sierra Leone, Liberia (Baize et al., 2014) and lastly Nigeria, Senegal and Mali. From the past, epidemics have occurred in the Democratic Republic of Congo, Sudan, Gabon, Republic of Congo and Uganda (Smith, 1978; Le Guenno et al., 1995, 1999).

Marburgvirus

The other member of the *Filoviridae* family is *Marburgvirus*, the silent cousin of Ebola. The virus Marburg was named after Marburg in Germany, but originated from Uganda, in Central Africa. Vervet monkeys [*Chlorocebus aethiops* (Gray, 1821)] importation for research purpose in Marburg and Belgrade (formerly Yugoslavia) brought the virus to these countries in 1967 (Smith et al., 1967). The first manifestation of *Marburgvirus* in Africa was a sporadic and fatal case, documented in Johannesburg, South Africa, in February 1975 from an Australian who came from Zimbabwe. Marburg hemorrhagic fever epidemiology will be discussed below. Ebola and Marburg viruses occurred in Africa, and at a much lesser extent in a primatology research center, in Manilla, Phillipines where *Reston Ebolavirus* (REBOV) has been described from cynomologus monkeys (*Macaca fascicularis* Raffles, 1821) imported into America (Philadelphia, 1989; Alice, Pennsylvania, 1990, 1996) and Italy (1996) (Rollin et al., 1999; WHO, 1992).

Epidemiology of Filoviral hemorrhagic fevers

Ebola hemorrhagic fever (EHF) or ebola virus disease (EVD)

EHF (EVD, International Classification of Diseases, ICD-10) is of major public health concern in the rural areas of sub-Saharan Africa, where *Ebolavirus* reached human population, after escaping its sylvatic foci first, then spread into rural/urban areas where it caused deadly hemorrhagic manifestations in human population. Multiple *Ebolavirus* species are co-circulating in endemic areas and the emerging zoonosis remains one of the most important zoonotic viral diseases of human in sub-Saharan Africa, because there is no approved treatment

and no licensed vaccine. EVD outbreaks occurred sporadically in Africa, scattered, within 10° latitude of the equator (Peterson et al., 2004; Groseth et al., 2007). This area is of dense and humid rainforest, characterized by succession of two rainy seasons and two dry seasons, providing the ecological niches favorable for *Ebolavirus* spp. amplification, maintenance and circulation.

It is likely that the vertebrate animals involved in *Ebolavirus* circulation find the optimal conditions necessary for sheltering, feeding and breeding and that the factors modulating *Ebolavirus* emergence are associated with those ecosystems. Spatio-temporal distributions of human *Ebolavirus* spp. outbreaks in Africa have already been well documented and mapped (Peterson et al., 2004; Pourrut et al., 2005; Groseth et al., 2007; Changula et al., 2014; Rougeron et al., 2015). *Ebolavirus* epidemics arose generally at the same time of the year (end of the dry season-beginning of the rainy season), when reservoir species of the virus gather with other sensitive hosts because of scarcity of food source, modification of ecological habitats which imply encroachment of different vertebrate animals. Also, population dynamic over time (physiological status such as reproduction time, demographic explosion of sensitive naive species) and space (migration) might conduct to amplification and emergence of *Ebolavirus*.

Ebolavirus dissemination

When the optimal conditions for *Ebolavirus* spp. circulation into those ecosystems are met, their probability to escape from these foci is enhanced. Peterson et al. (2004) used an ecologic niche modeling of outbreaks and sporadic cases of filovirus-associated hemorrhagic fever (HF) to provide a large-scale perspective on the geographic and ecologic distributions of Ebola and predicted that EVD would occur in the humid rain forests of central and western Africa. They observed that filovirus' transmission to humans is not common, and most occurrences can be traced to a single index case (WHO, 1978), followed by a spillower reaching the population. The following hypotheses can be considered for the introduction of the virus to nonhuman primate populations: 1) Non-human primates might have shared and eaten fruit rests containing virus in residual bat saliva and directly infected themselves. Gonzalez et al. (2007) theorized this pathway, stating that chronically *Ebolavirus* spp. infected bats might drop down partially eaten and masticated fruit spats or pulp picked from the canopy to the ground, promoting indirect transmission of the virus to some terrestrial dwelling mammals. Viral particles shed in bat saliva infected by the way, infect the rests of fruits secondly eaten by ground mammals. It has been shown that females chimpanzees mostly gave some collected fruit to their depending offspring and that adult male share meat with females and juveniles (de Wall, 1989); 2) Infected individuals can contaminate their group during care and social behavior, 3) Great apes also hunt and share other primates preys such as vervets, galagos and colobes and can be infected with contaminated meat. Assessing that infection of primates colonies begin with a single index case is then more difficult to support. Several individuals can contract the virus at the same time and contribute to disseminating it, because of their social behavior, 4) Natural secretions such as feces, urine, body fluid, placental rest and secretion might be shed in nature and represent a potential source of contamination to other small terrestrial mammals. Great apes and forest duikers fed on fruit rests become infected and might later represent the first link of a human transmission chain if rural communities enter into contact with those wild animals, via hunting. It is an epidemiological schema that might transpose the virus in a human population.

Olival and Hayman (2014) summarized, in their proposed transmission dynamic, that chiropteran are the potential reservoirs maintaining an intra-interspecies *Ebolavirus* circulation, and transmitting it to non-human primates and forest duikers; while direct transmission to human as well as rodents and pigs remain to be elucidated. Also, there is no yet evidence that wild animals, excepted non-human primates, can transmit directly the virus to human populations. The role of mosquitoes in their transmission model is questionable, interhuman transmission via natural secretions favors the virus spreading. Bausch et al. (2007) tested several body fluids as saliva, stool, semen, breast milk, tears, and nasal blood and concluded that EBOV is shed in a wide variety of bodily fluids during the acute period of illness but that the risk of transmission from vomits in an isolation ward and from convalescent patients is low. Humans can transmit the virus as soon as symptoms appear and continue to be infectious during the later stages of the disease as well as after death. Burial ceremonies in which mourners have direct contact with the body of the deceased person can also play a role in the transmission. *Ebolavirus* has been detected in semen for up to 82 days, and *Marburgvirus* for up to 13 weeks (Martini and Smith, 1968; Bausch et al., 2007), after the onset of illness, suggesting that these viruses could be eventually transmitted by sexual route (Bausch et al., 2007).

Analyzing the origin of contaminations

After the first Ebola outbreaks that occurred between 1976 -1979 (DRC and Sudan), the second waves of *Ebolavirus* spp. epidemics occurred between 1994-1997, after a silent period of 15 years; a first case was linked to a chimpanzee autopsied by a Swiss ethnologist in Ivory Coast, West Africa, and was attributed to a new strain, CIEBOV. The Kikwit epidemic (DRC), Mekouka, Mayibout and Booue (Gabon) were due to ZEBOV reemergence (Amblard et al., 1997; Georges et al., 1999). The source was a deep forest gold-mining camp, suggesting that

workers of the mine entered the reservoir/vectors biota. Mayibout outbreak was related to Mekouka's epidemic. Booue epidemic also began by an infected hunter who accidentally entered the sylvatic cycle at this time, while a high viral sylvatic amplification was going on as suggested by died chimpanzees that tested positive for *Ebolavirus* infection. From 2000 to 2004, multiple epidemics were recorded and attributed to ZEBOV at the border of Gabon and the Republic of Congo and to SEBOV in Sudan and Uganda, affecting simultaneously large populations of gorillas and chimpanzees (Leroy et al., 2002, 2004b; Bermejo et al., 2006). The first findings that the Swiss ethnologist was infected by a chimpanzee and the fact that the Mayibout outbreak originated in deep forest and was related to a gold-mine, drew the schema of an implication of forest mammals, more specifically cave dwelling mammals. ZEBOV remerged in 2005 in the Republic of Congo, in 2007-2009 in Democratic Republic of Congo, twelve years after the 1995 Kikwit outbreak. Two successive epidemics arose in the Luebo region (Kasai Occidental Province, DRC) in 2007 and 2008 and were caused by *Zaire ebolavirus* (Grard et al., 2011). Phylogenetic analyses performed on the full-length genomes of the two Luebo strains revealed that they were nearly identical, but not related to the lineage including ZEBOV strains from the 1976-1996 outbreaks (DRC and Gabon), nor to the descendants of the lineage including animal-derived sequences since 2001 and the human strains from the Mbandza-Mbomo 2003 and Etoumbi 2005 outbreaks (Gabon-RDC), with which they do, however, share a common ancestor (Grard et al., 2011). The Luebo 2007 outbreak represented an independent viral emergence, favored by a viral spillover caused by a dispersed reservoir species. Like the 1994-1997 Gabonese epidemics, these cross-border outbreaks were concomitant to marked wildlife epizootics (Leroy et al., 2004b; Rouquet et al., 2005; Lahm et al., 2007).

Chimpanzees, gorillas and duikers were susceptible hosts responsible for viral introduction into human populations. SEBOV emergence was also recorded in Uganda from 2011-2012, as in the DRC in 2012 (http://www.cdc.gov/vhf/ebola/resources/outbreak-table.html). In their modeling of geographic distribution of filovirus disease across Africa, Peterson et al. (2004) predicted the eastern extreme as the predilection area of *S. ebolavirus*, but this species emerged in DRC, the viral spillover being probably favored by widely dispersed reservoirs. In the past decades, in particular, FHFs incidences have increased and have been seen in areas they were not reported previously. Before, FHFs have never been recorded in Guinea until December 2013 when the first cases arose in the Southeast (Baize et al., 2014). Ebola virus disease was spreading unrecognized, while typical hemorrhagic fever cases such as Lassa fever or yellow fever, endemic in the area, were suspected but not proven. The hemorrhagic disease has been spreading quietly until late March 2014 when the diagnosis was finally confirmed Ebola virus disease. Human to human transmission via contact of fluids favored a spillover and the disease reached the neighboring countries of Sierra Leone and Liberia bordering the original epicenter of the outbreak. Lastly, the outbreak reached unexpected proportion in two months (Baize et al., 2014; Gire et al., 2014; Pigott et al., 2014; Wauquier et al., 2015), overwhelming the fragile health system in those developing West African countries. The epidemic touched the cities of Conakry (Guinea), Freetown (Sierra Leone), Monrovia (Liberia), Lagos (Nigeria), Dakar (Senegal) and Kayes (Mali), reaching the specter of a regional, even international dissemination. In fact, imported cases have been noticed in the USA (Dallas, Texas; Chevalier et al., 2014), Spain (Madrid; Parra et al., 2014) and the United Kingdom (London; Kuhn et al., 2014). Also, contaminated healthcare workers have been transferred to Hamburg (Germany) and Lyon (France) for care. The disease spread from Central Africa to West Africa. Among the known *Ebolavirus* species, four have emerged in sub-Saharan Africa, causing deadly outbreaks: *S. ebolavirus* (SEBOV), *Ivory Coast ebolavirus* (ICEBOV), *Bundibugyo ebolavirus* and *Z. ebolavirus* (ZEBOV) recently incriminated in the biggest Ebola epidemic ever recorded. The forested area of Guinea has been the epicenter and the source of contamination is discussed subsequently. While the Guinean EVD outbreak was spreading in the neighboring countries of West Africa, Ebolavirus reemerged in July 26, 2014, for the seventh time, in Democratic Republic of Congo, in Inkanamongo village, in the vicinity of Boende town (Equateur province). A total of 69 cases were reported, including 8 cases among health care workers, with 49 deaths (Maganga et al., 2014). A coding-complete genome sequence of EBOV that was isolated during this outbreak showed 99.2% identity with the most closely related variant from the 1995 outbreak in Kikwit (DRC) and 96.8% identity to EBOV variants that are currently circulating in West Africa (Maganga et al., 2014). The two outbreaks were in fact caused by two novel EBOV variants, consensually named Makona (West Africa) and Lomela (Middle Africa), after the Makona River close to the border between Liberia, Guinea and Sierra Leone and the Lomela River that runs through DRC's Boende District, respectively (Kuhn et al., 2014). The genetic characterization of the virus, combined with the geographic location of the outbreak, demonstrate that the DRC outbreak is an independent event, without any epidemiologic or virologic connection with the continuing epidemic in West Africa (Kuhn et al., 2014; Maganga et al., 2014).

Marburg hemorrhagic fever (MHF) or Marburg viral disease (MVD)

Marburgvirus was described from the Behring laboratory,

in Marburg, Germany from Vervet monkeys (*Chlorocebus aethiops*) imported from Uganda (Smith et al., 1967). Infected monkeys presented typical hemorrhagic fever clinical tables (Jahrling et al., 1990; Peters et al., 1992). That first Marburg outbreak reported with severe viral hemorrhagic fever was related to the handling of organs and tissues from those green monkeys (Smith et al., 1967; Martini, 1969). Eight years later, the first manifestation of *Marburgvirus* in Africa happened, in Johannesburg, South Africa, in February 1975, sporadic and fatal. It concerned an Australian just returning from a trip to Zimbabwe where he slept frequently in the open and once in an abandoned house which loft was inhabited by numerous bats (Gear et al., 1975). The third recognized Marburg manifestation affected a French engineer in Kenya in 1980 that subsequently infected his doctor before dying. He visited the Kitum cave (Mont Elgon National Park) where large populations of bats were sheltering. Next, another Marburg case has been reported and concerned a Danish who died after visiting the Kitum cave in August 1987 (Kenyon et al., 1994). After a silent period of more than 30 years, Marburg virus, the long neglected Ebola virus relative, called for attention in its cradle of Central Africa, hitting twice recently, and in large proportion: 1) 1998-2000, a gold-mining community in Durba, in the northeastern region of the Democratic Republic of the Congo, was affected with a high mortality rate reaching 83% (Rec, 1999; Baush et al., 2006); 2) 2004 and 2005, a second and large Marburg outbreak followed in northern Angola (West Africa), in the province of Uige (Rep, 2005; Towner et al., 2006) with a mortality rate higher than that during the 1998-2000 outbreak of Durba above cited (Towner et al., 2006).

Surprisingly, an Ebola outbreak was expected because of the large area affected reaching a big community since a first single infected case working in a gold-mining company. In July and September 2007, miners working in Kitaka Cave, Uganda, were diagnosed with MHF (Towner et al., 2009). At the same time (June-September 2007), 4 miners from Ibanda District contracted MHF through exposure to bats secretions in a mine in Kamwenge District, Uganda (Adjemian et al., 2011). Genetically diverse viruses isolated from tissues of the Egyptian Fruit Bat as well as detection of RNA MARV from these bats supported that *Rousettus aegyptiacus* was responsible for the epidemic. In late 2007, an American tourist contracted MVD in the python cave and in July 2008, another tourist from Netherlands was also infected with MARV in the same cave, from which diverse genetically MARVs were also isolated from *R. aegyptiacus* (Amman et al., 2014). Confined in a jungle cycle as *Ebolavirus*, *Marburgvirus* emerged and expressed its pathogenic potential, such as that one for *Ebolavirus*, without any doubt. As for *Ebolavirus* epidemics, *Marburgvirus* outbreaks in Africa were also well mapped and documented (Bausch et al., 2006; Feldmann, 2006; Brauburger et al., 2012; Rougeron et al., 2015). Imported human cases of Marburg virus infection from Uganda have been also reported in the USA (Timen et al., 2009) and in Netherlands (Fujita et al., 2010). Practically, all MARV emergences have been related to bat shelters (caves, gold-minning areas) and contact with infected monkeys (Cercopithecidae). These events clearly traced back the source of contamination to chiropters and primates Cercopithecidae. Both filoviruses are afrotropical, originally infectious of fruit bats (Chiroptera, Pteropidae) that seem playing the major role in their epidemiology, namely their maintenance and circulation in nature that will be discussed in a comparative manner in this review. *Ebolavirus* emerged mostly than *Marburgvirus*, but in terms of epidemiology both filoviruses are very similar. They share bats as the same vertebrate hosts.

Clinical manifestations and pathology of Ebola and Marburg viral diseases

At several times that a FHF arose in Africa, other endemics diseases such as Lassa fever, Yellow fever, malaria, cholera or typhoid fever were suspected. That has been the case for this ongoing Ebola epidemic in West Africa, where local Guinean healthcare workers attributed the first reported hemorrhagic cases to Lassa fever (Vogel, 2014). In 2007, the RDC ZEBOV emergence was also concomitant to an epidemic of typhoid and shigellosis. Then, the clinical table of filovirus-infected patients is non-specific and difficult to separate from other endemic diseases. The asymptomatic incubation period of filoviruses is 2-21 days. Symptoms usually manifest abruptly by a fever (greater than 38.6°C), severe headache, muscle pain and malaise. Secondly, severe diarrhea, nausea, vomiting, respiratory disorders, abdominal pain and weakness appear, accompanied with a lack of appetite. Hemorrhagic manifestations are observed in 30-50% of patients and vary in severity. Spontaneous abortion has been recorded within pregnant woman (Baize et al., 2014; Vogel, 2014). The pathogenesis of these hemorrhagic fevers includes necrosis of many organs, particularly liver (Martines et al., 2014). It has been suggested that the hemorrhages and shock manifestations may be a consequence of endothelial cell infection, with consequent loss of endothelial integrity leading to rapid hypovolaemic shock, multiple effusions and bleeding (Fisher-Hock et al., 1985). Death ensues within few days but some infected people recover.

However, patients who die usually have not developed a significant immune response to Ebola infection. *Z. ebolavirus, S. ebolavirus, Bundibugyo ebolavirus* and *Forest ebolavirus* cause severe illness in humans, although Forest virus infections have rarely been documented. *Reston ebolavirus* does not seem to be pathogenic for humans, but people may seroconvert after exposure to infected nonhuman primates or pigs.

Infection with *Marburgvirus* develops an acute illness for up to three weeks at least, accompanied by the following signs and symptoms: fever, generalized body pain, nausea and vomiting, headache, anorexia, malaise, abdominal pain, diarrhea, dyspnea, dysphagia, hiccups, conjunctivitis, rash or petechiae and abnormal bleeding from the nose, mouth, gastrointestinal tract, or genitourinary tract (Bausch et al., 2006). Death arises within few days, but as for EVD, some MVD infected people recovered.

The reservoir search

Several investigations targeting different vertebrate animals have been undertaken to identify the natural vertebrates that host and lurk Ebola virus in nature, after the first emergences. Arata and Johnson (1977) tested 100 specimens from 501 vertebrates collected in 1977 from Sudan; Germain (1978) screened more than 800 bedbugs and 147 mammals in DRC; Breman et al. (1999) collected 1664 animals of 117 species around the areas where the 1976 Ebola hemorrhagic fever occurred in the DRC and in Cameroon; Leirs et al. (1999) screened 3000 animals primarily from forest areas near the home of the index case after the Kikwit Ebola epidemic (DRC). Samples were representative of the different class of mammalia, reptilia and birds; even plants were suspected and tested. Globally, no evidence of *Ebolavirus* infection was found. Swanepoel et al. (1996) conducted experimental inoculation of thirty-three varieties of 24 species of plants with *Z. ebolavirus*, no evidence of infection was observed. Vertebrate animals inoculated included pigeons, young snakes, rodents, laboratory mice colonies, tortoises, lizards, frogs, toads and bats. Two microchiroptera of the family Molossidae, the Angola free-tailed bat, *Tadarida condylura* and the little free tailed bat, *Tadarida pumila* and one megachiroptera of the family Pteropidae, the Wahlberg's epauletted fruit bat, *Epomophorus wahlbergi* were able to asymptomatically replicate the ZEBOV with high viral titers, 4 weeks after inoculation, demonstrating for the first time that bats might be reservoirs hosts of *Ebolavirus* (Swanepoel et al., 1996). Invertebrates as cockroaches, leafhoppers, spiders, social ants, myrmicine ants, millipede and land snails were also inoculated but did not yield any proof of virus replication (Swanepoel et al., 1996). Turrell et al. (1996) negatively tested the ability of three mosquitoes *Aedes albopictus, Aedes taeniorhynchus* and *Culex pipiens* (Diptera, Culicidae), and one soft tick, *Ornithodoros sonrai* (Ixodida, Argasidae) for *Ebolavirus*. Arthropods have never been successfully infected following inoculation (Swanepoel et al., 1996, Turell et al., 1996), although several observations suggest they can transmit Ebola virus to humans, as demonstrated by Kunz et al. (1968) who showed that Marburg virus persist for more than 3 weeks in *Aedes* mosquitoes after experimental inoculation. Since their first emergences in 1976 (Ebolavirus in Yambuko, RDC and Nzara, Sudan), and in 1975 (*Marburgvirus* in Johannesburg, South Africa), natural reservoirs of filoviruses remained elusive for 3 decades and any investigation was not able to reveal where these viruses persist in nature, during inter-epidemic periods until 2005 when Leroy et al. (2005) provided the first evidence of bats as possible natural reservoirs.

The first documented primary infections of natural MVD outbreaks in Africa have been linked to human visiting caves inhabited by bats: gold mining in Kitaka Cave in the Kamwenge District, Uganda (Adjemian et al., 2011); visit of python Cave in Maramagambo Forest Uganda (Fujita et al., 2010; Timen et al., 2009). These findings provided the first clues that bats might play an important role in the transmission cycle of MVD (Monath, 1999; Peterson et al., 2004; Bausch et al., 2003), and evidence of MARV circulation in bats was only been documented when Towner et al. (2007) first detected MARV nucleic acids and antibodies from the common Egyptian fruit bat, *Rousettus aegyptiacus* in 2002 and 2005 in Gabon, without any virus islation. Swanepoel et al. (2007) also found MARV nucleic acid and antibody to the virus in the serum of insectivorous and fruit bats trapped in the Goroumbwa Mine, in northeastern DRC, but their attempts to isolate the virus were unsuccessful. Later, Towner et al. (2009) isolated MARV nine months apart from Egyptian fruit bats of the Kitaka cave in Uganda, demonstrating long-term virus circulation among the bat reservoir species. Genome sequences of MARV isolated from bats closely matched those isolated from miners during this epidemic, indicating that common Egyptian fruit bats represent major natural reservoir and source of Marburg virus with potential for spillover into humans. Despite the isolation of MARV from naturally infected Egyptian fruit bats captured in the Kitaka cave near Ibanda, in Western Uganda (Towner et al., 2009) and the python cave in the Queen Elisabeth National Park, Uganda (Amman et al., 2014), experimental inoculation of *R. aegyptiacus* with MARV were conducted and showed that the species is a natural reservoir host for MARV and demonstrated routes of viral shedding via rectal and oral routes capable of infecting humans and other animals (Amman et al., 2015). While the *Marburgviruses* exhibit high overall genetic diversity (up to 22%), only 6.8% nucleotide difference was found between the West African Angolan viruses and the majority of East African viruses, suggesting that the virus reservoir species in these regions are not substantially distinct. Remarkably, few nucleotide differences were found among the Angolan clinical specimens (0 to 0.07%), consistent with an outbreak scenario in which a single (or rare) introduction of virus from the reservoir species into the human population was followed by person-to-person transmission with little accumulation of mutations. This is in contrast to the 1998 to 2000 *Marburgvirus* outbreak, where evidence of several virus

genetic lineages (with up to 21% divergence) and multiple virus introductions into the human population was found (Towner et al., 2006).

Wild vertebrate hosts sensitive to Filoviruses

With the exception of *Reston ebolavirus,* all African filoviruses cause severe illness in nonhuman primates and some other animals. While there is no formal evidence for a causative role in some species, *Ebolavirus* outbreaks have been linked to reports of massive die-off of gorilla (*Gorilla gorilla*) and chimpanzee (*Pan troglodytes*) populations. An outbreak of Ebola decimated in November 1994, 25% of a wild chimpanzee community of 43 members in the Taï National Park, in Ivory Coast (Formenty et al., 1999), as did another in great apes of Minkebe Forest, north-eastern Gabon and in western equatorial Africa (Huijbregts et al., 2003; Walsh et al., 2003). Between 2001 and 2003, the epidemics that occurred in Gabon and Republic of Congo were also, for the first time, linked to concurrent animal mortality, mainly gorillas, chimpanzees and duikers (Leroy et al., 2004b; Bermejo et al., 2006). Detection of EBOV infected corpses in these three species strongly incriminated *Ebolavirus* as the causative agent.

Their population decreased and duikers were estimated to have fallen by 50% between 2002 and 2003 in the Lossi sanctuary, Republic of Congo, while chimpanzees lost 88% of their populations (Leroy et al., 2004b). *Ebolavirus* was also incriminated in a marked decline in gorilla and chimpanzee populations in the same areas, at the same point in time in Mekouka and Mayibout outbreaks. Small EBOV-specific genetic sequences were amplified from organs of six mice (*Mus setulosus* and *Praomys* sp., Rodentia, Muridae) and a shrew (*Sylvisorex ollula*, Insectivora, Erinaceidae), in Central African Republic and provided the first documented biological evidence of EBOV presence in healthy animals (Morvan et al., 1999), however this data was not sufficient enough, to attribute a reservoir status to these animals, being given lack of specific serologic responses, nucleotide specificities in the amplified viral sequences, failure of virus isolation, and the non-reproducible nature of the results. *Ebolavirus* infects a large variety of animal species, as attested by exploration of dead wild animal carcasses analyses. During the Gabon and RC epidemics (2001- 2004), the remains of animals were found in the surrounding forest (Rouquet et al., 2005). Thirty four samples taken from those carcasses (bones, muscles and skin) were analyzed using a panel of highly sensitive techniques, such as reverse-transcription polymerase chain reaction (RT–PCR), serology, histopathology and immunohisto-chemistry (IHC). Fourteen of them (10 gorillas, 3 chimpanzees and 1 duiker) tested positive for Ebola infection, indicating that these three animal species can be naturally infected by EBOV.

Most infected animals probably died rapidly, as suggested by the rapidly fatal nature of experimental EBOV infection in a variety of non-human primate species (Pourrut et al., 2005). Analyses of animal carcasses show that the great apes of the central African forests are particularly at risk for Ebola. This was confirmed by a serologic survey based on 790 samples taken from about 20 primate species in Cameroon, Gabon and Republic of Congo (Leroy et al., 2004a). Interestingly, some positive samples largely preceded the first human outbreaks in these regions, suggesting a viral sylvatic amplification chronologically happening before human contact with the virus. The results suggest that these animals are in regular contact with the EBOV reservoir, that some of them survive the infection, and that EBOV has probably been present for a very long time in the central African forest region. EBOV-specific antibodies were also found in other monkey species such as mandrills (*Mandrillus* sp.), vervets (*Cercopithecus* sp.), baboon, and drills suggesting that EBOV circulation between Cercopithecidae may be very complex, and some of their representative might be amplifying hosts because some great apes developed an Ebola viremia after eating their congeners Cercopithecidae. *Ebolavirus* epidemiology might involve other reservoir/amplifying hosts' species different to bats, and the passage of the virus to gorillas and chimpanzees might be more complex than a simple direct contact from the main reservoir. It is also possible that there are several reservoir species, and that many other animal species are susceptible to the virus and thereby participate in the natural EBOV life cycle (Figure 1). These include duikers (forest antelope, *Cephalophus dorsalis,* Onguligrades, Artiodactyla, Bovidae) and bush pigs (red river hog, *Potamochoerus porcus,* Onguligrades, Artiodactyla, Suidae). Overall, non-human primates of the family Cercopithecidae (colobus, baboons, mandrills, vervets and guenons) seem less sensitive to *Ebolavirus* infection as compared to non-human primates of the family Hominidae (chimpanzees and gorillas).

The Egyptian fruit bat is the potential reservoir of MARV. Marburg virus has been circulating in this species between the python cave and the Kitaka cave in Uganda as suggested by virus' isolation obtained by Towner et al. (2009) and Amman et al. (2014). The fact that Marburg and Belgrade epidemics were caused by *Chlorocebus aethiops* imported from Uganda support a typical reservoir role of this green monkey for the virus Marburg. In fact, the monkeys that carried the virus to Europe in 1967 were kept on Lake Victoria island, in a holding facility where large numbers of fruit bats were sheltering (Swanepoel et al., 2007). Uganda represents a "hotspot" for MARV circulation. It's actually known that transmission cycle can be schematized as presented in Figure 2.

Chiropteran as probable natural reservoirs of filoviruses

Enquiries were carried out in Central Africa, aiming to

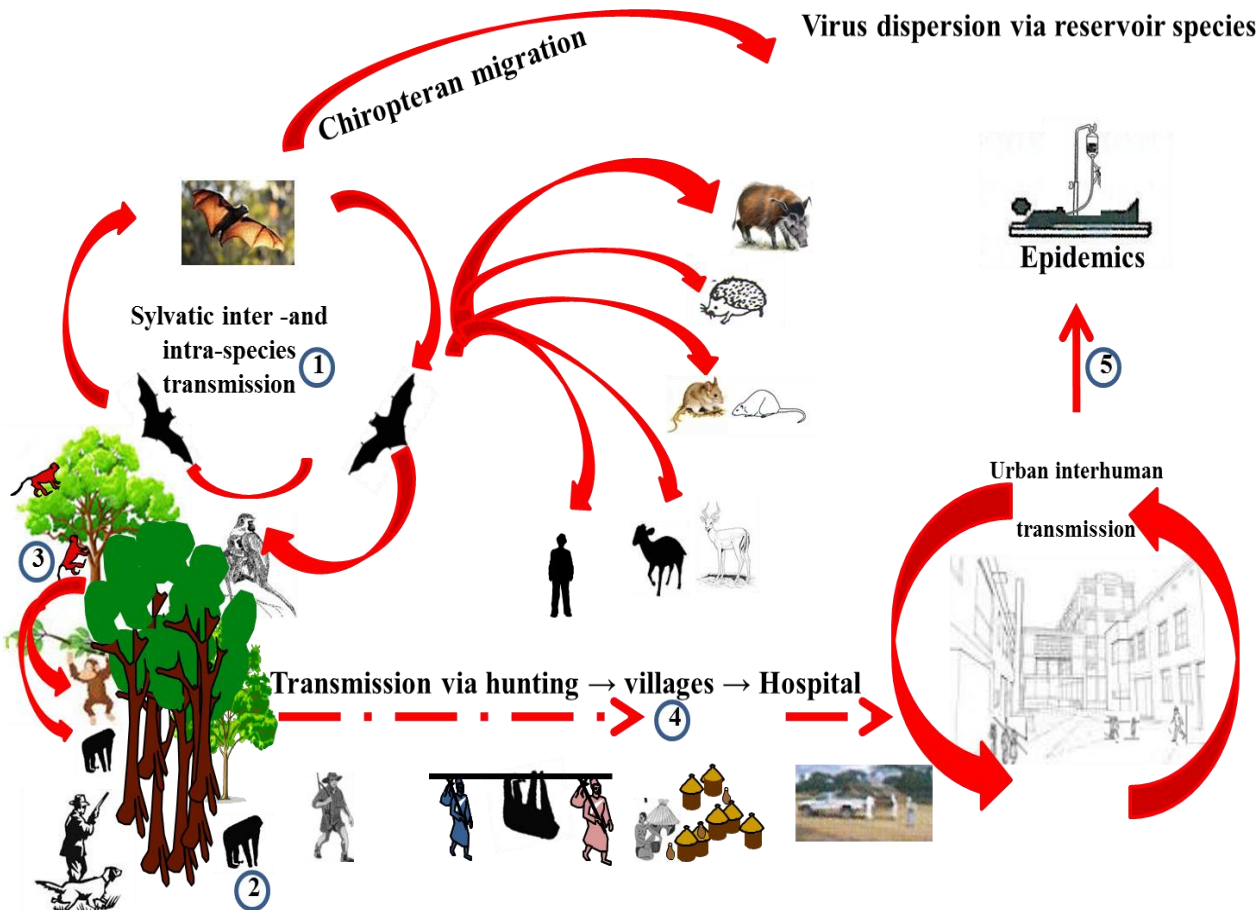

Figure 1. Ecoiagram of *Ebolavirus* transmission in nature. Fruit bats infected with Ebolavirus partially eat wild fruits in the forests (1). Partially chewed fruit contain virus particles enrobed in bat's saliva and dropped down from trees, contaminate other ground animals such as rodents, Insectivora, Onguligrades and non-human primates (2). Infected bats and Cercopithecidae are also eaten by great apes that are subsequently infected (3). Man can also be infected after intrusion in the canopy (caves and bat shelters) receiving directly bat's secretion infected with Ebolavirus. Mostly, hunting and handling of bushmeat (4) transposed ebolavirus from a sylvatic to an rural/urban transmission cycle causing deadly epidemics (5).

identify the natural reservoirs species of filoviruses (Leroy et al., 2005; Gonzalez et al., 2007; Pourrut et al., 2009). They found that bats belonging to the family Pteropidae were the major susceptible population, asymptomatically infected by the virus as attested by antibodies and viral nucleic acid detection. Serological studies conducted allowed to detect specific anti Ebola IgG from 16 bats: 4 Hammer-headed Fruit Bat, *Hypsignathus monstrosus* H. Allen, 1861, 8 Franquet's Epauletted bat, *Epomops franqueti* Tomes, 1860 and 4 Little Collared Fruit bat, *Myonycteris torquata* Dobson, 1878 (Chiroptera, Pteropidae) (Leroy et al., 2005; Gonzalez et al., 2007; Pourrut et al., 2009). Their studies also detected viral nucleic acid sequences in the tissues of 13 bats (3 *H. monstrosus,* 5 *E. franqueti* and 5 *M. torquata*) and provided the first evidence of bats' role as probable potential reservoirs of *Ebolavirus* in nature (Table 1). Swanepoel et al. (2007) investigated the reservoir hosts for Marburg virus (MARV) after the epidemic that hit the gold mining-community in Durba and detected MARV viral nucleic acid sequences from two insectivorous bats, the Greater Long-fingered Bat, *Miniopterus inflatus* Thomas, 1903 and the Eloquent horseshoe bat, *Rhinolophus eloquens* K. Anderson, 1905 (Microchiroptera, Rhinolophidae), and the Egyptian fruit bat, *Rousettus aegyptiacus* E. Geoffroy, 1810 (Megachiroptera, Pteropidae). Serological evidence of MARV circulation was detected by ELISA in *R. eloquens* and *R. aegyptiacus*. They concluded that these bats were implicated in *Marburgvirus* circulation around the Goroumbwa mine and its immediate surroundings. Towner et al. (2007) detected MARV-specific RNA, IgG antibody from *R. aegyptiacus* and isolated MARV for the first time from this species in Gabon, acting now as a typical reservoir of *Marburgvirus* (Towner et al., 2007). Pourrut et al. (2009) documented that both Ebola and

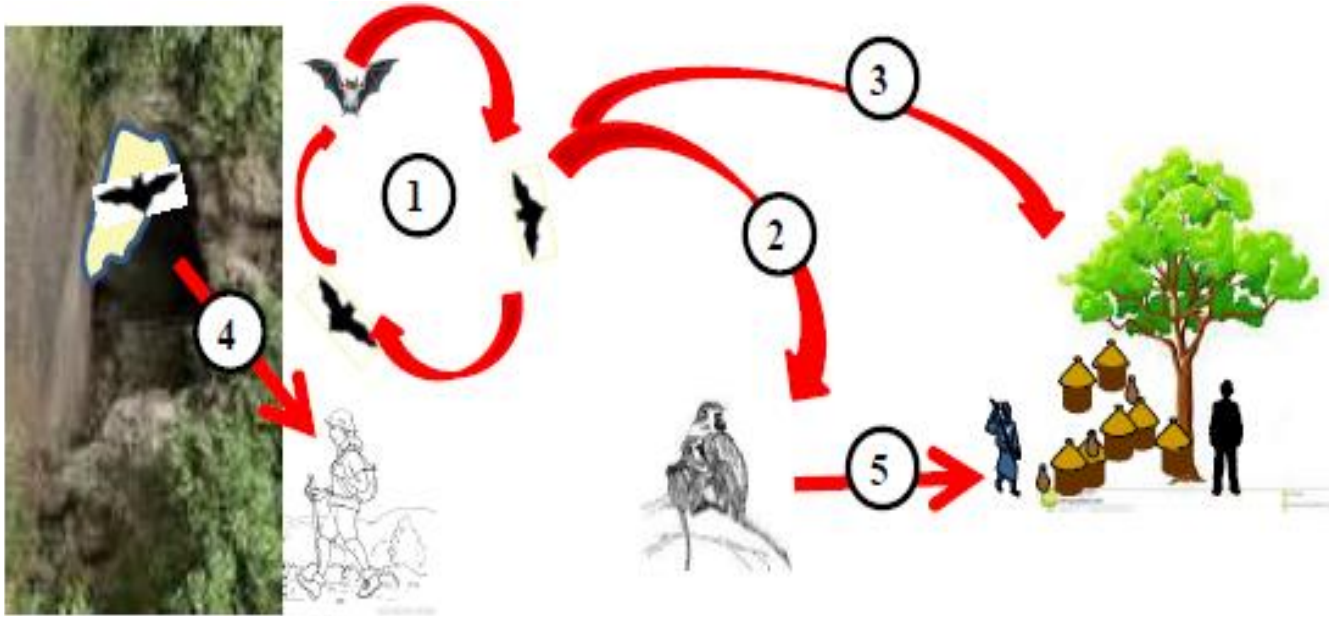

Figure 2. Ecodiagram of *Marburgvirus* transmission in nature. High intra-interspecific contact in roost facilitates rapid transmission of MARV between bats (1). Partially chewed fruit containing virus particles shed in bat's saliva and dropped down from trees, contaminate Cercopithecidae (2) and Hominidae (3). Man can also be infected after intrusion into the sylvatic (caves and bat shelters) receiving directly bat's secretion infected with MARV (4). Handling of monkeys tissues also directly infect human beings (5).

Marburg viruses co-circulated within the Egyptian Fruit Bat. Hayman et al. (2010) detected Zaire EBOV (ZEBOV) antibodies in a single Straw-colored Fruit Bat, *Eidolon helvum* Kerr, 1792 (Megachiroptera, Pteropidae) from a roost in Accra, Ghana; another fruit bat *Epomophorus gambianus* Ogilby, 1835 (Megachiroptera, Pteropidae) has been found infected with *Ebolavirus* by Hayman et al. (2012), as well as *E. franqueti* and *H. monstrosus*. Serological evidence of EBOV antibodies has been also detected in a serum sample of the Little flying Cow, *Nanonycteris veldkampii* Matschie, 1899 (Megachiroptera, Pteropidae) (Hayman et al., 2012). ZEBOV-IgG were detected again in *E. franqueti, H. monstrosus, R. aegyptiacus* and *M. torquata*; while the Lesser Epaulet bat, *Micropteropus pusillus* Peters, 1867 (Megachiroptera, Pteropidae) and *Mops condylurus* Lesson (Microchiroptera, Molossidae) tested for the first time ZEBOV-IgG positive in nature (Pourrut et al., 2009). MARV-IgG were also found in *R. aegyptiacus* and *H. monstrosus* (Pourrut et al., 2009). Amman et al. (2012) investigated the Python Cave inhabited by the Egyptian Fruit Bat in Uganda and detected viral nucleic sequences of MARV; also seven of the bats yielded Marburg virus isolates (Table 1). Using an enzyme-linked immunosorbent assay based on the viral glycoprotein antigens, Ogawa et al. (2015) detected IgG ZEBOV, and MARV in serum samples collected from the fruit bats (*Eidolon helvum*) in Zambia during 2006-2013. Distinct specificity for *Reston ebolavirus*, so far known only from Philippines and China, in Asia (Barrette et al., 2009; Pan et al., 2014), has been shown also from *E. helvum* for the first time in Zambia (Ogawa et al., 2015). Serological evidence of antibodies directed against flaviviruses and detection of viral nucleic acid incriminate those chiropters as potential reservoirs of filoviruses in nature. The isolation of MARV in nature supports a typical status of *Marburgvirus* reservoir species for *R. aegyptiacus.* Overall, these findings suggest a closer follow-up of the other bats, particularly of the family Pteropidae that can play the major role. Researches on the role of bats as reservoirs of filoviruses, particularly *Ebolavirus* are still ongoing, several vertebrate animals as Great apes and duikers are naturally infected by this virus, probably directly from the reservoir, but the pathways of its emergence in human environment is not yet fully understood. However, the epidemiological scenario so far advanced, make bats the most probable reservoir candidates for filoviruses.

Domestic vertebrate animals sensitive to filoviruses

Dogs and pigs are the only domestic animals so far identified as species that can be infected with EBOV. A survey conducted in Gabon on dogs eating dead animals showed over 30% seroprevalence for EBOV during the Ebola outbreak in 2001-2002 (Allela et al., 2005). Dogs asymptomatically incubate the virus; while pigs experimentally infected with EBOV can develop clinical disease, depending on the virus species. Pigs were experimentally able to transmit Zaire-Ebola virus to naive pigs and macaques; however, their role during Ebola

Table 1. *Marburgvirus* (MARV) and *Zaire ebolavirus* (ZEBOV), antibodies (IgG), and viral RNA sequences detected from bats in Africa.

Date	Bat species	Vernacular name (Order, Family)	Filovirus isolated				Filoviral event	
			Ebolavirus		Marburgvirus		Locality	Reference
			Antibodies detected	RNA sequences	Antibodies detected	RNA sequences		
January 2008	*Eidolon helvum*	Straw-colored Fruit Bat	IgG	PCR (-)	-	PCR (-)	Ghana Zambia*	Hayman et al., 2010 Ogawa et al., 2015*
May-June 2007	*Epomophorus gambianus*	Gambian Epauleted Bat (Megachiroptera, Pteropidae)	IgG	PCR (-)	-	PCR (-)	Ghana	Hayman et al., 2012
June 2003-March 2008	*Epomops franqueti*	Franquet's Epauletted Bat (Megachiroptera, Pteropidae)	IgG	PCR (**+**)	IgG	PCR (-)	Gabon, Ghana*	Pourrut et al. (2005, 2007; 2009) Hayman et al., 2012*
May-June 2007	*Hypsignathus monstrosus*	Hammer-headed Fruit Bat (Megachiroptera, Pteropidae)	IgG	PCR (**+**)	IgG*	PCR (-)	Gabon*, RC*, Ghana	Pourrut et al., 2009* Hayman et al., 2012
June 2003-March 2008	*Micropteropus pusillus*	Lesser Epauleted Bat (Megachiroptera, Pteropidae)	IgG	PCR (**-**)	IgG	PCR (-)	Gabon	Pourrut et al., 2009
June 2003-March 2008	*Myonycteris torquata*	Little Collared Fruit Bat (Megachiroptera, Pteropidae)	IgG	PCR (**+**)	-	PCR (-)	Gabon, RC	Pourrut et al., 2009
June 2003-March 2008	*Hyposideros gigas*	Giant Leaf-nosed Bat (Microchiroptera, Hypossideridae)	-	PCR (-)	IgG	PCR (-)	Gabon, RC	Pourrut et al., 2009
June 2003-March 2008	*Mops condylurus*	Greater Mastiff Bat (Microchiroptera, Molossidae)	IgG	PCR (**-**)	IgG	PCR (-)	Gabon	Pourrut et al., 2005; 2007; 2009
May-October 1999	*Miniopterus inflatus*	Greater Long-fingered Bat (Microchiroptera, Vespertilionidae)	-	PCR (**-**)	-	PCR (**+**)	DRC	Swanepoel et al., 2007
May-October 1999	*Rhinolophus eloquens*	Eloquent Horseshoe Bat (Microchiroptera, Rhinolophidae)	-	PCR (**-**)	IgG	PCR (**+**)	DRC, Gabon*	Swanepoel et al., 2007 Pourrut et al., 2009*
June 2003-March 2008	*Nanonycteris veldkampii*	Little flying Cow (Megachiroptera, Pteropidae)	-	PCR (**-**)	IgG	PCR (**-**)	Ghana	Hayman et al., 2012
May-October 1999	*Rousettus occidentalis*	Egyptian Fruit Bat (Megachiroptera, Pteropidae)	-	PCR (**-**)	IgG	PCR (**+**)	RDC	Swanepoel et al., 2007
May-June 2007	*Rousettus occidentalis*	Egyptian Fruit Bat	IgG	PCR (**-**)	IgG*	PCR (**-**)	Ghana, Gabon*, DRC*	Hayman et al., 2010 Pourrut et al., 2009*
June 2003-March 2008	*Rousettus occidentalis*	Egyptian Fruit Bat	IgG	PCR (**+**)	IgG	PCR (**-**)	Gabon, Republic of Congo	Pourrut et al., 2009

Table 1. Contd

2005-January 2006	*Rousettus occidentalis*	Egyptian Fruit Bat	-	PCR (**+**)	Virus isolation	PCR (**+**)	Gabon*	Towner et al. (2007 ; 2009)*
Aug 2008-Nov 2009	*Rousettus occidentalis*	Egyptian Fruit Bat	-	PCR (-)	Virus isolation	PCR (**+**)	Uganda	Amman et al., 2012
June-July 2007	*Rousettus occidentalis*	Egyptian Fruit Bat	-	PCR (-)	-	PCR (**+**)	Kenya	Kuzmin et al., 2010

When several documented filoviral events happened in different localities, the mark on the locality's name refer to the author with the same mark. Republic of Congo (RC), Democratic Republic of Congo (DRC).

outbreaks in Africa needs to be clarified (Weingartl et al., 2013). In 2009 Reston-EBOV was the first EBOV reported to infect swine with possible transmission to humans (Weingartl et al., 2013).

ECOLOGY OF BATS AS POTENTIAL RESERVOIRS OF FILOVIRUSES

Hypsignathus monstrosus, *Epomops franqueti* and *Myonycteris torquata* approximately share the same vital domains, the two last species being sympatric (Pourrut, 2007). They are confined to the tropical Central Africa and extent their distribution range to the wetter part of West Africa (Figure 3). They are found natively along and on either side of the equator, between latitudes 10°N and 10°S. They have been also recorded eastwards to Uganda and southwards to Angola and Congo (Rosevear, 1965). *H. monstrosus* is the less gregarious species among these; living in companies of a maximum of 20 individuals hanging close together daily up in trees or low down in shrubs. The Hammer-headed Fruit Bat has a preference for the closed forest what affiliate it to the Guinean woodlands where it finds dense patched of forest, with a variety of fruits maturing successively over seasons. Rosevear (1965) postulated that a little is known about its mode of life. Dispatched records of *H. montrosus*' occurrence have been noted, but nobody gave information about its migration range north and south the equator according to the season. Other bioecological features related to mating, breeding, feeding and roosting are not well known. Sanderson (1940) recorded a little colony of the Hammer-headed Fruit Bat resting into rocks, what seems unusual in current scientific literature, the species might have switched to a tree sheltering bat, because of scarcity of cave-dwelling structures. The Franquet's Epauletted bat, *E. franqueti*, occurs in West Africa, from Ghana to Loanda in Angola, and across the continent to the great Lakes as far south as Tanganyika. As the Hammer-headed Fruit Bat, it is a closed forest species and does not appear to be gregarious too; only few specimens have been found roosting together, hanging freely from trees or low bushes (Rosevear, 1965). Its bioecological features are not also well known. The Little Collared Fruit bat, *M. torquata*, shares the same predilection areas as the previous two other Ebola probable reservoirs, but a little is known about its habits (Rosevear, 1965). *R. occidentalis*, a potential filovirus reservoir species, is common and widely distributed in Africa (Figure 4). Its migration range can lead to a large variety of epidemiological situations. Over the ten species of the genus *Rousettus* known worldwide, *Rousettus occidentalis* is the mostly represented in Africa, numbering several subspecies, *R. a. arabicus* of the Arabic Peninsula (Saudi Arabia, Yemen, Oman, Pakistan, Iran), *R. a. aegyptiacus* in Egypt, Turquia, Syria, *R. a. unicolor* in West Africa, *R. a. leachi* in East, *R. a. angolensis* (or *Lissonycteris angolensis*) from Guinea to Kenya and from South Angola to Zimbabwe and *R. a. princeps*, *R. a. tomasi, R. a. unicolor* on the islands of Guinea gulf. The genus *Rousettus* is widely distributed and colonizes a large range of areas including dry and humid ecosystems, within altitudes reaching 4000 m. It is the only megachiroptera actually found roistering into caves and treeholes, thousands of individuals can also shelter into roofs of non-occupied human habitations, bridges. Bats of the genus *Rousettus* leave their shelter at sun down and fly around 30 km for feeding. A little is known about their migratory behavior (1 individual has been caught 500 km far away from its previous shelter in South Africa few days after). Widely common in sub- Saharan Africa (Figure 5), *Eidolon helvum* live in large colonies reaching 1, 000, 000 individuals of both sex (Walker, 1999), hanging on trees, often in cities. This fruit bat is of interest in *Ebolavirus* epidemiology because of its wide range migration, reaching more than 2,500 km (Richter and Cumming, 2008). The typical predilection area of the Straw-colored Fruit Bat is the forested areas of Central Africa where it is

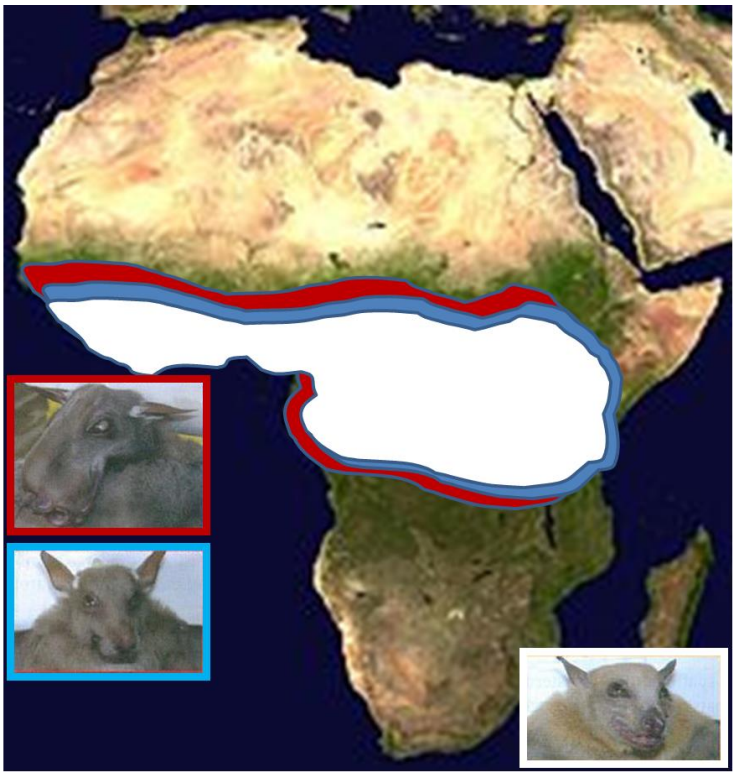

Figure 3. Distribution of *Hypsignsthus monstrosus* (red), *Epomops franqueti* (white) and *Myonycteris torquata* (blue) in Africa. The vital domains of the three species are overlapping.

Figure 4. Distribution of *Rousettus aegyptiacus occidentalis* in Africa.

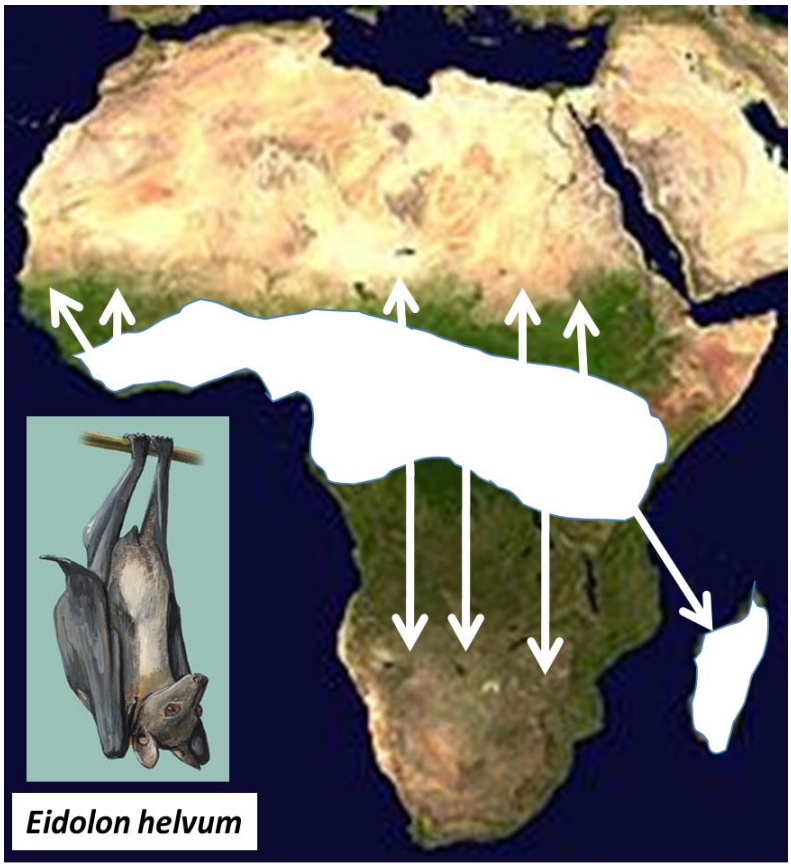

Figure 5. Distribution of *Eidolon helvum* in Africa. Arrows indicate their migration routes.

present year-round but its migration routes conduct numerous colonies of the fruit bat to North and South of Africa. Anderson (1907) reported its distribution from Somalia, Djibouti, southeastern Ethiopia and Sudan in the northeast; Senegal, Gambia and Mali in the northwest, to Malawi, South Africa and Zimbabwe in the south. The transition of filovirus species causing outbreaks in Central and West Africa during 2005-2014 seemed to be synchronized with the change of the serologically dominant virus species in the species *E. helvum* (Ogawa et al., 2015), but surveillance programs seem too limited over time and space to state that the serological status of these bats has changed. *Epomophorus gambianus*, contrarily to the other Pteropodids suspected to be reservoirs of *Ebolavirus*, is not associated with the forested areas of Central Africa. Indeed, the Gambian Epauleted bat prefers open grasslands, woodlands and savannah of Western Africa (Figure 6). It has been recorded in the forest edges, and occurs from Senegal to Southern Sudan and Ethiopia (Rosevear, 1965). The Sahel Acacia-wooded grassland and deciduous bush land form its northern limit of predilection. Its particular ecological features might involve it in a less manner in *Ebolavirus* ecology; in fact the species roosts singly or in groups of a maximum of 50 individuals (Rosevear, 1965), and does not compete with the other known Ebola potential reservoirs. *N. veldkampi* migrates northward from the forest of Ivory Coast and into the savannah during rainy season. They can fly 500 km and roost in small groups of well-spaced individuals (Reeder, 1999). Plurispecific associations have been noted between bats of the genus *Rousettus* and other microchiroptera such as the Giant Leaf-nosed Bat, *Hipposideros gigas* (Wagner), the Benito Leaf-nosed Bat, *Hippossideros beatus* K. Anderson, 1906 and the High-crowned Bat, *Miniopterus inflatus* (Thomas, 1903) in Gabon (Pourrut, 2007). Considering that ecological feature, an eventual role of microchiroptera as reservoir or amplificatory hosts of filoviruses needs to be investigated. In fact, Saez et al. (2015) recently suspected that *M. condylurus* might be involved in the zoonotic origin of the ongoing 2013-2015 West African EVD epidemic. The Eloquent horseshoe bat, *Rhinolophus eloquens* is found in Eastern Africa (Ethiopia, Kenya, Rwanda, Somalia, South Sudan, Tanzania and Uganda). This cave dwelling microchiroptera is associated with natural habitats of the subtropical or tropical moist lowland forests, dry savanna and moist savanna. The Greater Long-fingered Bat, *Miniopterus inflatus* is a

Figure 6. Distribution of *Epomophorus gambianus* in Africa.

species inhabiting high forested areas where they roast in colonies reaching 1000 of individuals in caves, crevices and rocks sometimes in association with other insectivorous bats as *Hypossideros caffer* or fruit bats as *Lyssonycteris angolensis*. It is common in Central Africa (Cameroon, Gabon, Central African Republic, Democratic Republic of the Congo, Equatorial Guinea, Uganda) and East Africa (Ethiopia, Rwanda, Tanzania and Kenya). It has been recorded in West Africa (Guinea, Liberia) and south to Africa (Mozambique, Namibia and Zimbabwe). Epidemiological scenari can be amplified by a response to environmental modifications, often resulting from human activities. *Ebolavirus* amplification in nature has been documented by Pourrut (2007) who found that it was correlated with reproduction time, changing from a country to another because of climatic specificities. *Hypposideros gigas*, *Mops condylurus*, *Miniopterus inflatus*, and *Rhinolophus eloquens* are the microchiroptera so far suspected as potential reservoirs of *Ebolavirus* spp. They proliferate in most of the African biota south to Sahara and in the island of Madagascar, of the Indian Ocean. Generally, microchiropters are not migratory bats. Their seasonal movements are not well studied but seem to be local. The four microchiropters so far found associated with *Ebolavirus* in nature are present between the latitudes 10°N and 10°S, on both sides of the equator. Occurrences areas of *H. gigas* and *R.s eloquens* almost overlap (Figures 7 and 8), covering the western central part of Africa; while some dispatched records are noted for *M. inflatus* which share the same ecosystems with the two precedents (Figure 9). This species has been recorded in Ethiopia, Uganda, Kenya and Tanzania in East Africa; and from Namibia, Zimbabwe and Mozambique in southern Africa. The predilection areas of *Mop condylurus* are much larger; this species is widely distributed over much of sub-Saharan Africa, ranging from Senegal, Gambia and Mali in the west, to the Sudan, Ethiopia and Somalia in the east (Figure 10). It has been also recorded southwards through much of eastern and southern Africa, and Swaziland. The species appears to be largely absent from the Congo Basin (Figure 10). As most of the microchiropters, they eat insects that abound in greater or less profusion all year long under the tropics (Rosevear, 1965). Involved in the filoviruses' epidemiological cycle, microchiropters will then maintain local enzootic cycles of

Figure 7. Distribution of *Hypposideros gigas* in Africa.

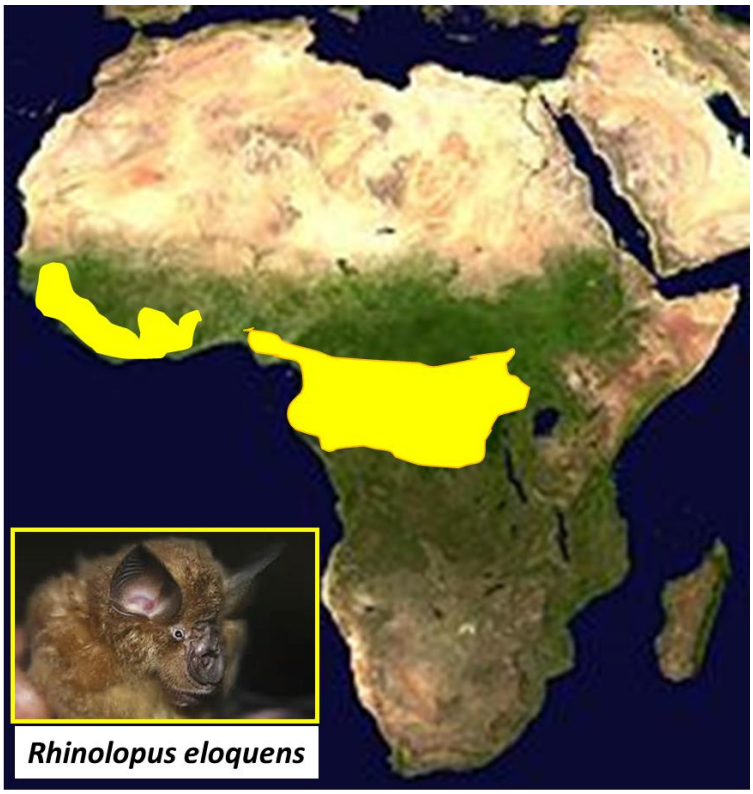

Figure 8. Distribution of *Rhinolopus eloquens* in Africa.

Figure 9. Distribution of *Miniopterus inflatus* in Africa.

Figure 10. Distribution of *Mops condylurus* in Africa.

infection and play an important role in the perpetuation of filoviruses within ecosystems.

The microchiropters, at the opposite of megachiropters which include the single family of Pteropidae, account for fifteen different families known worldwide among which eight have an Afrotropical biogeographical distribution: Emballonuridae, Megadermatidae, Molossidae, Myzopodidae (Malagasian Subregion),Nycteridae, Rhinolophidae/Hipposideridae, Vespertilionidae (http://planet-mammiferes.org). Rosecvear (1965) noticed that they breed at most times of the year, though there are indications of preferences for the dry season.

INVESTIGATION OF THE ZOONOTIC ORIGIN OF FILOVIRAL HEMORRHAGIC FEVERS

The natural source of the first Ebola outbreaks occurring from 1976 to 1979 has never been elucidated despite several research tentative targeting different vertebrate animals (Breman et al., 1999; Germain, 1978; Arata and Johnson, 1977; Leirs et al., 1999). Later, the Swiss ethnologist's infection with *Ebolavirus* was related to a chimpanzee she was autopsying (Le Guenno et al.,1995). Similarly, the 1996 Mayibout outbreak in Gabon originated from children who found and butchered a chimpanzee in the forest (Georges et al., 1999). Similar sources have been reported for Marburg virus which caused the 1967 outbreak in Marburg and Belgrade linked to the handling of organs and tissues of *C. aethiops* monkeys imported from Uganda (Smith et al., 1967; Martini, 1969). Practically all the sources of *Ebolavirus* outbreaks in Democratic Republic of Congo and Gabon were related to animal carcasses of gorillas, chimpanzees and duikers, hunted and handled since the forest (Olloba, 2001; Grand-Etoumbi, 2002; Entsiami 2002; Yembelengoye, 2002; Leroy et al., 2004b) as well as for the epidemics of Etakangaye 2001, Olloba 2002, Mendemba 2001, Ekata 2001 and Mvoula 2003. The presence of bats were recorded several times in the warehouses of the cotton factory, where the first people infected during the 1976 and 1979 outbreaks in Nzara, Sudan were working. No other likely source of infection was identified in either outbreak. It is also noteworthy that the Australian who was infected by Marburg virus (and subsequently infected two other people in Johannesburg in 1975) had just returned from a trip to Zimbabwe, during which he had slept frequently in the open and once in an abandoned house, the loft of which was inhabited by numerous bats. A few days before becoming ill, the French engineer who was infected by Marburg virus in Kenya in 1980 (and who subsequently infected his doctor) had visited caves containing large bat populations (Smith et al., 1982). However, when baboons and Vervet monkeys were placed in cages inside the same caves, none became infected (Johnson, 1996 personal communication), the experience might be set up into the caves out of the virus' amplification period in bats, or monkeys were resistant to infection and had developed an immunity following a previous contact with the virus. The fact that bats have already been implicated as source of infection in some previous filovirus outbreaks such as the Marburg hemorrhagic fever outbreak of Durba (Democratic Republic of Congo) inspired the IRD Research Unit 178 (Fundamentals and Domains of Disease Emergence) and opened the way to investigation of an eventual role of bats as reservoirs of those mysterious filoviruses. Swanepoel et al. (1996) experimentally proved that the Angola free-tailed bat, *Tadarida condylura* and the little free tailed bat, *Tadarida pumila* (Microchiroptera, Molossidae) and the Wahlberg's epauletted fruit bat, *Epomophorus wahlbergi* (Megachiroptera, Pteropidae), were able to asymptomatically replicate ZEBOV with high viral titers, 4 weeks after inoculation, but the first attempts to isolate the virus from bats in nature were not successful (Germain, 1978; Arata and Johnson, 1977; Breman et al., 1999; Leirs et al., 1999). The mystery was dissipated when an IRD (UR 178) team based at the CIRMF first discovered that bats of the family Pteropidae might be involved in replication, incubation and filoviruses (Ebola and Marburg) maintenance and transmission in nature (Pourrut et al., 2005; Leroy et al., 2005; Towner et al., 2007) and enhanced future directions for the research on reservoir species. Hypothetical transmission routes that seem plausible are proposed (Gonzalez et al., 2007; Olival and Heyman, 2014); however more investigations are needed to elucidate the ways that filoviruses borrow from the reservoir to nonhuman primates and to humans. While the struggle for containing the deadly EVD outbreak in West Africa was going on, few studies searched to figure out where it came from, and what was its zoonotic carrier. It is hypothesized that the ongoing EVD epidemic originated from a little 2 years old girl who might have been infected by *Eidolon helvum* in Guekedou (Funk and Piot, 2014). There has been no handling or consummation of bush meat in the village, the toddler might have collected a partially chewed fruit dropped from a tree by the straw-colored fruit bat and subsequently became infected with virus particles in residual bat saliva (1st hypothesis). Saez et al. (2015) investigated the zoonotic origin of the West African *Ebolavirus* outbreak around Meliandou where the toddler first contracted the ZEBOV strain, but did not find any evidence of virus circulation in wildlife. Particularly, bats belonging to the incriminated species (*E. helvum*) that were captured and tested did not allow any virus isolation or ZEBOV sequences detection. Also, their enquiries conducted on wildlife did not reveal any decline of sensitive wild animals, but observed that there was a tree with large hollow in the index home, inhabiting microchiroptera among which *M. condylurus* has been identified. This insectivorous bat already tested ZEBOV-IgG positive (Pourrut et al., 2009) and might be the source

of the infection, because kids usually caught and played with bats in this tree (2nd hypothesis). Free-tailed bats have been already incriminated in such infection as for the first Sudan Ebola virus outbreaks (World Health Organization/International Study Team, 1978). Cases of Marburg virus infection via exposure to bat colonies have been already documented with the Kitum cave in Mont Elgon National Park, Kenya, and in Zimbabwe. A total of 12 bats have been suspected to be potential hosts of Ebola and Marburg viruses in the Afrotropical biogeographic region (Table 1). They include 8 megachiropters of the family Pteropidae: *H. monstrosus, M. torquata* and *E. franqueti,* mostly associated with the forested areas as previously discussed. *E. gambianus, E. helvum* and *R. occidentalis* found positive for filoviruses have tested negative in June 2006, in Senegal supposed Ebola free and used as a control site (Pourrut, 2007), *M. pusillus* and *N. weldkampi*. 4 microchiropters are identified as probable reservoirs: *M. condylurus M. inflatus*, *H. gigas* [Pourrut et al. (2009) list it as IgG ZEBOV positif], and *R. eloquens*.

PLACE OF CHIROPTERS IN THE EPIDEMIOLOGY OF EMERGING ZOONOTIC DISEASES

Bats harbor a potential role as reservoirs for zoonotic diseases. About 66 different viruses have been isolated from bats (Calisher et al., 2006) and serological evidence for infection of bats with many viruses has been found (Kuno, 2001; Messenger et al., 2003; Gonzalez et al., 2008). Studies of their bioecology, dynamic and natural behavior have been enhanced from the 1970s since they have been incriminated in zoonoses' emergence due to coronaviruses, filoviruses and paramyxoviruses. They considerably participate on diseases dispersal across a vast range of regions where they are involved in the increasing threat of emerging infectious diseases to human societies: the severe acute Middle East respiratory syndrome-like coronavirus (MERS-CoV) (Ithete et al., 2013; Memish et al., 2013), paramyxoviruses Nipah virus (NiV) in Malaysia and Bangladesh (Luby, 2013), Hendra (HeV) in Australia (Clayton et al., 2013), and lyssavirus disease in America, Europe and Australia (Warrell and Warrell, 2004; Van der Poel et al., 2006) plus the emerging filoviruses, Ebola and Marburg in Africa (Leroy et al., 2005; Calisher et al., 2006). It has been already established that rabies virus infections in France have been associated with the migratory routes of the Nathusius' pipistrelle, *Pipistrellus nathusii* Keyserling and Blasius, 1839 (Brosset, 1990). In Africa, the widely separated geographic locations of Ebola outbreaks have supported that the reservoir and the transmission cycle are probably closely associated with the rainforest ecosystem, assertion supported by antibodies distribution. The fact that outbreaks seldom occur suggests the presence of a rare or ecologically isolated reservoir species having few contact with human and non-human primate species (Gonzalez et al., 2005). In the Class Mammalia of the vertebrate animals, the order Chiroptera represents the second in terms of species diversity, behind the order of Rodentia, but is the most important because of its potential for harboring zoonotic pathogens. It includes the suborders of Microchiroptera and Megachiroptera; the last accounting for the unique family of Pteropidae which include the Old World fruit bats or flying foxes found in tropical and subtropical Africa and east to the Western Pacific. Most of the actually suspected filoviruses' reservoirs belong to that family. The Microchiroptera are found throughout most of the world and include small insectivorous bats, few bat species fruit and flower feeders, few carnivorous bats, and lastly vampire bats which have a Neotropical geographic distribution, found in tropical areas of the American continent, principally in Mexico, Chile, Argentina and Brazil. Rodents are terrestrial and commensally mammals, closely associated with human environment and carry significant diseases with a real public health concern (Mills, 2006). As examples, Hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome are due to hantaviruses pathogens hosted by rodents of the family Muridae (Schmaljohn and Hjelle, 1997). Lymphocytic choriomeningitis, Lassa fever, Argentina, Bolivian, Venezuelan and Brazilian hemorrhagic fevers are caused by rodent's arenaviruses. These small mammals are also incriminated in Congo Crimean Hemorrhagic Fever and Rift Valley Fever epidemiology (Camicas et al., 1990; Pretorius et al., 1997). They become less studied than bats which do not directly interact with human environment, because they are phytophilous (associated with forest vegetation) or lithophilous (associated with caves, rocks and similar sheltering structures) (Rosevear, 1965). Compared with rodents, bats are unique in their propensity to host zoonotic viruses, they are natural reservoirs of a number of high-impact viral zoonoses. In their quantitative analysis, Luis et al. (2013) demonstrated that bats indeed host more zoonotic viruses per species than rodents, because their sympatry with other species of the same taxonomic order promote interspecific transmission and zoonotic viral richness.

THE PROBABLE ROLE OF ANIMALS INVOLVED IN FILOVIRAL HEMORRHAGIC FEVERS

In the light of reservoir species theory of Rodhain (1998), the following criteria can be considered: 1-Efficient vertebrate reservoirs (or good reservoirs) of filoviruses need to be receptive to these viruses, not just slightly sensitive. They must be able to asymptomatically replicate the virus, develop an efficient and sufficient viremia, and once infected, the animal must survive; ensuring maintenance and circulation of the virus in nature, and therefore the foci's continuity. 2- the reservoir species must be of an abundant and prolific population, able to replicate and disseminate the pathogen. Neonate

or naive individuals are non-immune, which allow their receptivity to the virus and infection, ensuring continuation. 3- The viremia must be of a high viral titer, last longer enough, the time to allow it to infect other receptive hosts of the same population for virus perpetuation.

In its natural foci, a filovirus circulates between several vertebrate hosts, playing different roles in its epidemiology. For Ebola and Marburg viruses, bats are the potential candidates for the reservoir status: 1) Filovirus RNA characterization associated with virus specific antibodies and virus isolation within some bats species provided clues that chiropters might be incriminated; 2) It is also likely that the reservoir species are ecologically isolated, associated with the rainforest ecosystem with an important potential of migration which might justify the scattered geographic occurrences of Ebola outbreaks. Bats satisfy this statement. Other vertebrates are just activating the foci for a while, acting as amplifying hosts: in this category, belong some monkeys of the family *Cercopithecidae* such as vervet, *Chlorocebus aethiops*, found infected with a filovirus in Marburg (Smith et al., 1967) and the red colobus, *Procolobus badius,* hunted and eaten by chimpanzees, who subsequently became infected by *Ebolavirus* (Boesch, 1994). The virus can also reach some other non-susceptible animals unable to replicate it or who just present a temporary short viremia with a low viral titer: the dead-end hosts. Birds that tested refractory to *Ebolavirus* (Swanepoel et al., 1996) must be listed in this category. Widely divergent orders or families of the avian fauna were unable to experimentally replicate *Ebolavirus*. Then, efforts on field reservoir search should focus more on other animals able to replicate the virus than birds. Migratory vertebrates will disseminate the virus: bats again fit in this case, spreading pathogens through migration; and other sensitive hosts will serve as sentinel hosts or biological markers, allowing the epidemiologists to detect the virus' activity. That's the case for great apes (chimpanzees and gorillas) which have a wide range of vital domain but do not move as far as migratory bats. Once in contact with the virus, they die, promoting about a probable emergence. Animals involved in a filovirus' activity are not necessarily all reservoirs. Incidental hosts are just accidentally involved in the cycle, like mosquitoes that might be infected after a viremic blood meal taken on a wild animal. In addition, filoviruses generally do not replicate in arthropods or arthropod cell lines (Peterson et al., 2004). Due to their dispersal, several index cases should be reported if anthropophilic mosquitoes were able to disseminate filoviruses. A filovirus can adopt several different reservoirs, in different environmental conditions; an animal species might be a good reservoir in a certain environment and a bad one in another. In the case of bats for example, food is found in some restricted areas, depending to the phenology of wild fruit trees, which varies from season to season (even month to month). If the availability of food is good, they stay around for several nights or even weeks, and chronically infected bats would increase the length of time during which they can infect other receptive species and are qualified as good reservoirs in such environmental conditions. If their survival conditions are not met, they must necessarily travel further afield and will not stay longer enough to perpetuate their carried pathogen in this specific ecosystem and are circumstantially qualified as bad reservoirs. Mostly wild vertebrates (birds and mammals) act as usual reservoirs for most of the pathogens. Domestic or commensal mammals, as well as human beings, are rarely involved as reservoirs. In the case of many arboviruses, arthropods are involved in their maintenance because of their longevity and their vectorial competence allowing them to replicate and transmit the virus through vertical transmission to the offspring. The bats might do the same for filoviruses, but will transmit the virus to the offspring through placental exchanges. In fact, Leroy et al. (2006) postulated that great apes might be contaminated while touching bat placental tissues and biological fluids, during parturition. Bat's ability for long distance flying provides an intensive selective force for coexistence with viral parasites through a daily cycle that elevates metabolism and body temperature analogous to the febrile response in other mammals (O'Shea et al., 2014). These factors imply a large diversity of epidemiological situations according to the virus, the bat reservoir species and the region. Understanding epidemiological situations need a comprehension of the evolution of these linked systems in correlation with the modification of ecosystems, often resulting from human induced activities on the environment. Repeated passages of filoviruses from a vertebrate host to another will, sooner or later, develop modifications of their viral genome in response to new environmental adaptation, by emergence of reassortants during coinfections. In such conditions two situations are predictable: 1- the virus might lose some virulence and this can lead to extinction of its foci, 2- after genome modification, the foci are activated after a short silent interval, increasing the ability of the virus to last longer. This last scenario happened in Sierra Leone and contributed to maintaining the virus' adaptation. In Fact, Gire et al. (2014) tracked *Ebolavirus*' evolution during this West African epidemic and found that it was changing as it spread. Their genetic analysis revealed that the outbreak in Sierra Leone was sparked by at least two distinct viruses, introduced from Guinea at about the same time. One of this disappearing from patients sampled later in the outbreak, while a third lineage appeared. Then, for several different reasons, it appears puzzling, to predict the ending of the outbreak because of those mutations, and to set efficient preventive measures axed at level of natural reservoirs.

FACTS, THEORY AND HYPOTHESIS

Zoonoses are diseases that originate from wildlife and

strike living animals, threatening animal biodiversity and public health (Daszak et al., 2000; Leroy et al., 2004a; Woolhouse et al., 2005; Lahm et al., 2007; Jones et al., 2008). Filoviral hemorrhagic fever asymptomatically develops in the wild vertebrate host and cause fatal manifestations when it reaches human beings (anthropozoonose). Filoviruses are circulating in a sylvan cycle among reservoir species and other sensitive hosts. EVD is an anthropozoonose, benign within the reservoir species, fatal within sensitive human population where it is associated with a mortality rate ranging from 50 (SIEBOV) (Smith, 1978; Baron et al., 1983) to 80% (ZEBOV) (Bwaka et al., 1999; Nkoghé et al., 2004), depending on the virus species (Johnson, 1978). The duikers and great apes (gorilla and chimpanzee) are also sensitive to *Ebolavirus* infection and represent intermediate hosts that can bridge the virus to human population. Humans entering the forest can be infected while hunting bats or other apes, antelopes and sensitive hosts. It is in that occasion that the virus reaches rural population, spreading from human to human, causing outbreaks and even epidemics affecting several villages and towns. These outbreaks can provide a source for potentially devastating urban epidemics, which are the most dangerous, because of concentration of susceptible people; typically higher mortality rates associated with urban situation are recorded after prolonged human-to-human transmission. However, the role of bats with their spectrum of behavioral variation, in the forested areas of central Africa where the virus originated from is unclear.

ECONOMIC AND SOCIAL IMPACT OF FILOVIRUSES EMERGENCE

The public health and economic burden imposed by FHFs on the developing world with limited medical coverage are enormous. The West African EVD outbreak caused global societal and economic impact due to the unexpected magnitude of the epidemic killing thousands of people; the socioeconomic impacts in Guinea, Sierra Leone and Liberia include job losses, smaller harvests and food insecurity. Travel, global business and other life activities were affected, taking a significant human toll as well as cause public fear, economic loss and other adverse outcomes. While the primary cost of this tragic outbreak is in human lives and suffering, the crisis will secondly worsen already entrenched poverty. The Bank Group estimates that Guinea, Sierra Leone and Liberia will lose at least US$1.6 billion in economic growth in 2015 (http://www.worldbank.org). As of April 2015, the World Bank Group's response to the Ebola crisis has mobilized US$1.62 billion to support the affected countries containing and preventing the spread of infections, providing treatment and care, and improving public health systems. They also mobilized funds for providing 10,500 tons of maize and rice to seed more than 200,000 farmers in Guinea, Liberia and Sierra Leone, averting hunger in Ebola-affected countries and reviving agriculture. In terms of morbidity and mortality, EVD accounts largely among the global disease burden of humankind. As of April 19, 2015, 23816 cases of EHF (14893 laboratory confirmed were reported, accounting for 10736 deaths in Guinea, Sierra Leone, Liberia and to a less degree, in Nigeria and Mali (http://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/index.html) (Figure 11). The bulk of FHF mortality occurs in sub-Saharan Africa where it is seeded by the lethal emergence of the most deadly *Ebolavirus* species, *Z. ebolavirus* (ZEBOV) and the existence of a wide range of potential bat reservoirs. Despite the rarity and ecologically isolation of the reservoir species, the force of FHF transmission in some areas of sub-Saharan Africa is extremely high (25,907 cases suspected, probable and confirmed), intensively driven by interhuman transmission. FHF are socially devastating diseases of the developing world and the risk of epidemics remains. Since the last emergence of ZEBOV in Gueckedou and Macenta, Southeastern Guinea (Baize et al., 2014), on December 2013, the disease continues to sicken and kill thousands of people in the affected countries of sub-Saharan Africa. It is difficult to control because of repetitive health care workers, medical doctors and laboratory diagnosis personnel direct contamination. Nosocomial infections occurred in the hospital, during the Yambuko epidemic (1976), a Belgian nuns inadvertently started the epidemic by giving vitamin injections to pregnant women, through reuse of unsterilized syringes, needles or other medical equipment contaminated with body fluids (Piot, personal communication). Inadequate dispositions for contact with Ebola infected patients throughout herbalist care, burial preparation, including body washing and long intimate funeral ritual greatly increased the risk of the virus spillover, by fluid transmission. By September 14, 2014, a total of 318 cases, including 151 deaths, had been reported among health care workers (WHO Ebola Response Team, 2014).

It is the first West Africa Ebola outbreak and the largest ever recorded in history; morbidity and mortality recorded are higher than in all previously Ebola outbreaks combined in Africa. This EVD epidemic is very similar to the 1976 outbreak. Both were caused by *Z. ebolavirus,* hitting rural forest communities first, before spreading into urban areas, without any link to bush meat handling. Hemorrhagic cases were suspected due to malaria, typhoid, Lassa fever, yellow fever or influenza. From the past, epidemics have occurred in the Democratic Republic of Congo, Sudan, Gabon, Republic of Congo and Uganda (Smith, 1978; Le Guenno et al., 1995, 1999). Filoviruses and mammals co-evolved since the Paleocene. The existence of orthologous filoviruslike elements shared among mammalian genera whose divergence dates have been estimated suggesting that filoviruses are at least tens of millions of years old (Taylor et al., 2010). Phylogenetic and sequencing evidence from gene boundaries was consistent with integration of filoviruses in mammalian genomes.

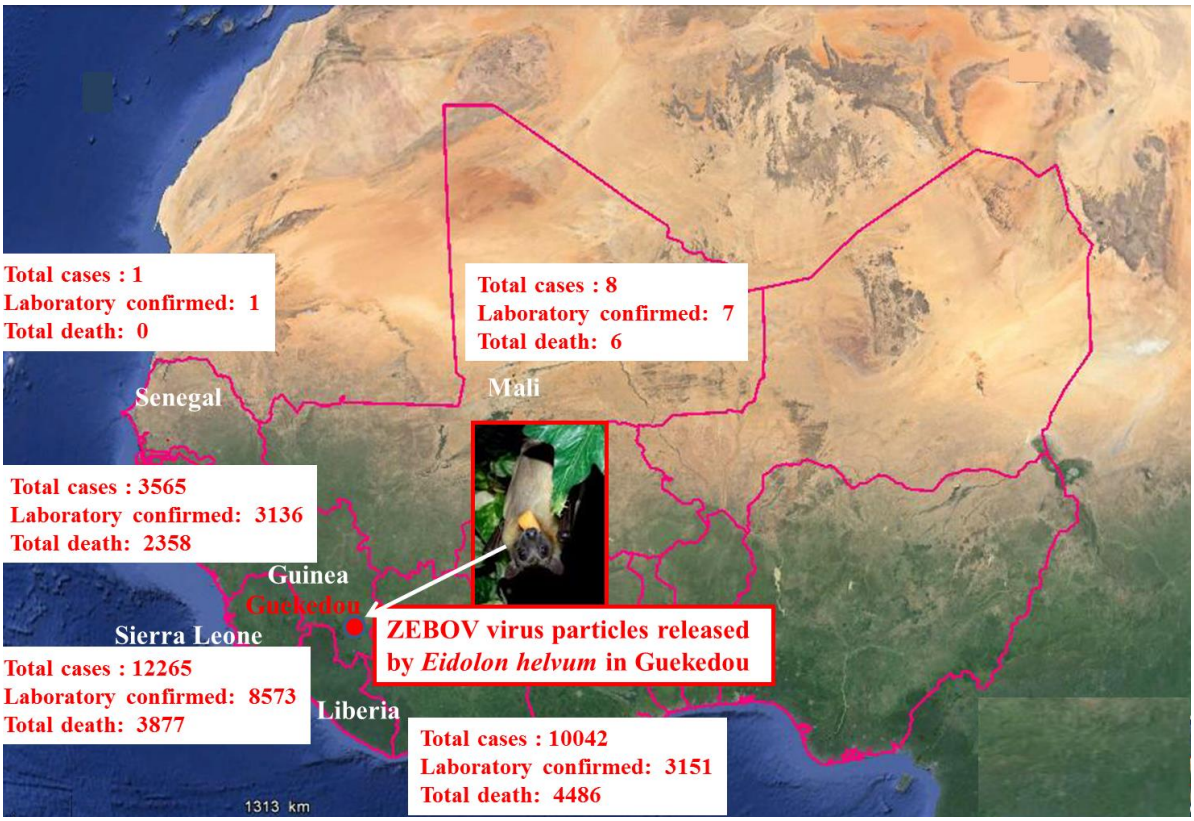

Figure 11. Incidence of ZEBOV activity in West Africa as at April 19, 2015.

FUTURE STUDIES

Despite the importance of the studies achieved on the epidemiology of filoviruses, a number of deficiencies have been pointed out and need to be addressed. A fundamental aim needs to assess the ecology of reservoirs in the rural/sylvan interface, where EVD transmission spills over into human populations. Filoviruses might silently breed in some West African forested ecosystems, introduced since the emerging areas of central Africa by some potential reservoirs as *E. helvum*. They can extend their amplification areas and reach other sensitive secondary hosts. Peterson et al. (2004) suggested that a large-scale ecologic and geographic comparison is an unexplored approach to identifying the natural reservoir of filoviruses in order to detect patterns of co-occurrence and co-distribution of viruses with potential hosts.

Studies extended to other Pteropidae sub families to see if any other potential reservoirs exist

Understanding the ecological features of the major suspected reservoirs of *Ebolavirus*, that is, *H. monstrosus*, *E. franqueti* and *M. torquata* is a major goal. Their principal known domains of occurrence is concern with the central forested areas of Africa, but some studies recorded *H. monstrosus* in Southern Senegal (Feiler, 1986; Koopman, 1975; Koopman et al., 1978), as well as *E. franqueti* and *M. torquata* (Pourrut, 2007). The roosting behavior of *R. aegyptiacus* needs to be investigated. Plurispecific associations have been observed among Pteropidae (Kunz, 1982; Kuzmin et al., 2010). Many bat species are gregarious, living in dense colonies: for example, *Eidolon helvum* aggregations can reach a population of 50,000 to 100,000 individuals per roost (Jones, personal communication; Rosevear, 1965). Roosting sites can also account for assemblages of multiple species where high intra and interspecific contact rates of bats from different origins and unknown pathologic and immune status directly promote rapid transmission of pathogens and their spread. The Egyptian Fruit Bat roosts daily in trees or caves, often with large groups of other bats. High-densities bat colonies have been observed, sometimes numbering in the thousands. They emerge from the roost to forage for food in the late evening, and return just before dawn. They hang upside down, with their wings folded closely around their bodies (http://en.wikipedia.org/wiki/Rousettus_aegyptiacus). We hypothesize that following those pluri-specific associations, competition for territory conquest or simply daily association into shelters might lead to infection of potential reservoirs

such as *R. aegyptiacus* which is known widespread in all the Afrotropical biogeographic region excepted the Saharan domain (Figure 4). A scenario such as this one might extend the known occurrence area of *Ebolavirus* since its natural foci of central forested African areas, *R. aegyptiacus* acting as the bridge vector. 1) Occurrence areas of the three known potential reservoirs (*H. monstrosus, E. franqueti* and *M. torquata*) need to be updated and mapped as well as for the other potential filoviruses reservoirs. In fact, several vector-borne, parasitic or zoonotic diseases have (re)-emerged and spread within Africa these recent years, because of global and local changes caused by either climate change, human-induced landscape changes like constant reduction in size of natural forests tending to make the original epidemiologic sylvatic cycle somewhat a relic one, switching to a rural cycle. This implies encroachment of people and livestock into wildlife habitats and in another direction increases wildlife migration from degraded areas into rural and peri-urban regions. Impacted landscape variation induced by environmental factors and human behaviors (hunting, irrigation; deforestation; cattle breeding...), added to climatic changes, directly impact human health. 2) Their dynamic over time (reproduction period) and space (migration) need to be completely understood for modeling the risk of *Ebolavirus* emergence. It has been already proven that most reservoirs are efficient *filovirus* vectors during sexual activity (reproduction time). In fact, Amman et al. (2012) observed that birthing seasons represent times of increased infection among juveniles and that most human MVD cases coincided with those periods. 3) Serologic studies undertaken along a West-East transect study across West Africa will assess to what extent the *Ebolavirus* amplification has been observed. Other Pteropidae close to the known reservoirs such as *Rousettus angolensis smithi*, *Eidolon* spp., *Micropteropus* spp., *Nanonycteris,* etc.., existing in Africa, need to be studied in order to discover other eventual filoviruses and bat reservoirs.

Migration routes and distribution areas of the potential bat species reservoirs

To fully understand their migration circuits and areas of predilection, the above cited transect study needs to be entirely prospected. The actually known EBOV serotypes might have circulating in a primeval cycle, among certain bat species (*Hypsignathus, Myonycteris*, *Epomops*…) without any symptomatic infection in the forest of Central Africa in a silent cycle. Man entering the forest gallery for the purpose of hunting might be occasionally involved in this cycle. Such a zoonotic reservoir of infection could exist in all forested areas (primary forest galleries, isolated patches of forest, forest-savanna mosaics) of West Africa. Ecosystems modification and environmental conditions linked to global change can influence spatial and temporal distribution and dynamics of human pathogenic agents. A high viral amplification of *Ebolavirus* in the forest ecosystem probably favoured its escape from its naturally sylvatic cot increasing the probability for the virus to reach directly human population or via other sensitive hosts. As shown by the phylogenetic study from Baize et al. (2014), the bottom clade contains *Ebolavirus* (ZEBOV) described from Gabon, suggesting that the other top clades derived from it. In fact, the derived clades show that ZEBOV emerged in DRC in 1976, simultaneously as SEBOV in Sudan, in 1976 before the Ivory Coast emergence of CIEBOV. Their ancestor, the Gabon strain (ZEBOV) emerged later in 1994, probably confined in a jungle cycle, before its emergence. All available data about the implication of bats in the epidemiology of EVD are limited to Central Africa, because the disease first emerged in this area. Little information is obtained from West Africa. Senegal is the extreme limit of the geographical range of the known Ebola reservoir species, that is, *H. monstrosus, E. franqueti,* and *M. torquata*. Ninety eight (98) bats belonging to the genus *Eidolon helvum, Epomoporus gambianus* and *Rousettus aegypticus occidentalis* were captured near Mbour (14°25' N, 16°57' W; MBour Dpt. [Thiès Reg.]), 80 km far away from Dakar in June 2006, and tested negative for EBOV (Pourrut et al., 2007). However, a serologic study of human and simian populations undertaken by Gonzalez et al. (2005) detected IgG from human population in Africa. The demonstration of neutralizing antibody to EBOV in the human sera suggested that there might be a sylvatic cycle of EBOV in West Africa. Marburg and Ebola viruses are endemic in Central African countries where outbreaks are unpredictable and just sporadically emerge.

Bioecology of the microchiropters, potential species reservoirs

Four species belonging to three different microchiropters' families (*Molossidae, Vespertilionidae*, and *Rhinolophidae*) are suspected for now in filoviruses' epidemiology. Some detailed studies need to be undertaken in order to clarify the following points: 1) are members of different families breeding at the same time of the year? 2) Do they successively breed over time? Responding to those questions will assess if seasonal amplification of a filovirus is short over time because of reproduction at the same period, with a sexual pause during which neonate bat species do not exist, corresponding to the inter-epizootic period. In the other case, the amplification period can last long and promoted by the opportunity of continuous contact of naive offspring with infectious bats in the colonies during a certain time of the year. This will conduct logically to a seasonal pulse of filoviruses in the ecosystem characterized by amplification periods separated by silent intervals. This scheme of amplification/silencing makes

sense if microchiropters were only proliferating in the ecosystem. Plurispecific associations include micro-chiropters and megachiropters, the last accounting individuals with large migration range (*Hypposideros* species and *R. aegypticus occidentalis* have been recorded together in the Kitaka cave, Uganda). Do both incubate filoviruses at the same time in nature? Are there reproduction/amplification periods synchronic? One might be a relay while another is entering a silent period. A comprehensive approach will investigate the natural reservoir of filoviruses which is large-scale ecologic and geographic comparisons in order to elucidate the patterns of (co) occurrence of viruses within potential hosts. Dynamic of the bat reservoir species of these filoviruses as well as interactions between sensitive hosts and bats in the rural/sylvan interface are not fully understood. Breman et al. (1999) conducted several researches aiming to identify the wild animal species hosting the virus in nature but failed to find the reservoirs. Extensive field and laboratory studies of the wide range of filoviruses activity in Central and West Africa need to be undertaken. The main emphasis will be the bioecology of the chiropteran with regard to the specific filovirus they carry. Sensitive serological assays need to be processed on a wide range of bats captured from diverse ecological forested areas as well as from other sensitive apes and Cercopithecidae in order to figure out the extent of the filoviruses amplification and dissemination. The 2013-2015 outbreak of EVD shows a higher fatality rate attributed to the strain ZEBOV, Quantitative Trait Loci maps of genetic factors that condition virulence of the Ebola strains isolated during these concomitant epidemics might be elucidated from a locality to another, and the already known Ebola virus strains so far isolated and incriminated during previous epidemics. Understanding the immune responses to filoviruses that ensure apathogenic, persistent infections in the reservoirs, without any sign of disease is a major goal.

Conflict of interests

The author(s) did not declare any conflict of interest.

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REFERENCES

Adjemian J, Farnon EC, Tschioko F, Wamala JF, Byaruhanga E, Bwire GS, Kansiime E, Kagirita A, Ahimbisibwe S, Katunguka F, Jeffs B, Lutwama JJ, Downing R, Tappero JW, Formenty P, Amman B, Manning C, Towner J, Nichol ST, Rollin PE (2011). Outbreak of Marburg hemorrhagic fever among miners in Kamwenge and Ibanda Districts, Uganda, 2007. J. Infect. Dis. 204(3):S796-799.

Allela L, Boury O, Pouillot R, Délicat A, Yaba P, Kumulungui B, Rouquet P, Gonzalez JP, Leroy EM (2005). Ebola virus antibody prevalence in dogs and human risk. Emerg. Infect. Dis. 11(3):385-390.

Amblard J, Obiang P, Edzang S, Prehaud C, Bouloy M, Le Guenno B (1997). Identification of the Ebola virus in Gabon in 1994. Lancet 349: 181-182.

Amman BR, Carroll SA, Reed ZD, Sealy TK, Balinandi S, Swanepoel R, Kemp A, Erickson BR, Comer JA, Campbell S, Cannon DL, Khristova ML, Atimnedi P, Paddock CD, Crockett RJK, Flietstra TD, Warfield KL, Unfer R, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Rollin PE, Ksiazek TG, Nichol ST, Towner JS (2012). Seasonal Pulses of Marburg Virus Circulation in Juvenile *Rousettus aegyptiacus* Bats Coincide with Periods of Increased Risk of Human Infection. PLoS Pathog. 8. doi:10.1371/journal.ppat.1002877.

Amman BR, Nyakarahuka L, McElroy AK, Dodd KA, Sealy TK, Schuh AJ, Shoemaker TR, Balinandi S, Atimnedi P, Kaboyo W, Nichol ST, Towner JS (2014). Marburgvirus resurgence in Kitaka Mine bat population after extermination attempts, Uganda. Emerg. Infect. Dis. 20(10):1761-4.

Amman BR, Jones ME, Sealy TK, Uebelhoer LS, Schuh AJ, Bird BH, Coleman-McCray JD, Martin BE, Nichol ST, Towner JS (2015). Oral shedding of Marburg virus in experimentally infected Egyptian fruit bats (*Rousettus aegyptiacus*). J. Wildl. Dis. 51(1):113-24.

Anderson K (1907). On *Pterocyon, Rousettus* and *Myonycteris*. Ann. Mag. Nat. Hist. 8 (1): 431-435.

Arata AA, Johnson B (1977). Approaches towards studies on potential reservoirs of viral haemorrhagic fever in southern Sudan (1977). In: Pattyn SR, Ebola virus haemorrhagic fever. Amsterdam: Elsevier/Netherland Biomedical. pp. 191-202.

Baize S, Pannetier D, Oestereich L, Rieger T, Koivogui L, Magassouba N, Soropogui B, Sow MS, Keïta S, De Clerck H, Tiffany A, Dominguez G, Loua M, Traoré A, Kolié M, Malano EM, Heleze E, Bocquin A, Mély S, Raoul H, Caro V, Cadar D, Gabriel M, Pahlmann M, Tappe D, Schmidt-Chanasit J, Impouma B, Diallo AK, Formenty P, Van Herp M, Günther S (2014). Emergence of Zaire Ebola Virus Disease in Guinea - Preliminary Report. N. Engl. J. Med. 371(15):1418-1425.

Baron RC, McCormick JB, Zubeir OA (1983). Ebola virus disease in southern Sudan: hospital dissemination and intrafamilial spread. Bull. WHO 61: 997-1003.

Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, Rollin PE, Towner JS, Shieh WJ, Batten B, Sealy TK, Carrillo C, Moran KE, Bracht AJ, Mayr GA, Sirios-Cruz M, Catbagan DP, Lautner EA, Ksiazek TG, White WR, McIntosh MT (2009). Discovery of swine as a host for the *Reston ebolavirus*. Science 325(5937): 204-206. doi: 10.1126/science.1172705.

Bausch DG, Borchert M, Grein T, Roth C, Swanepoel R, Libande ML, Talarmin A, Bertherat E, Muyembe-Tamfum JJ, Tugume B, Colebunders R, Kondé KM, Pirad P, Olinda LL, Rodier GR, Campbell P, Tomori O, Ksiazek TG, Rollin PE. (2003). Risk factors for Marburg hemorrhagic fever, Democratic Republic of the Congo. Emerg. Infect. Dis. 9(12):1531-1537.

Bausch DG, Nichol ST, Muyembe-Tamfum JJ, Borchert M, Rollin PE, Sleurs H, Campbell P, Tshioko FK, Roth C, Colebunders R, Pirard P, Mardel S, Olinda LA, Zeller H, Tshomba A, Kulidri A, Libande ML, Mulangu S, Formenty P, Grein T, Leirs H, Braack L, Ksiazek T, Zaki S, Bowen MD, Smit SB, Leman PA, Burt FJ, Kemp A, Swanepoel R (2006). Marburg hemorrhagic fever associated with multiple genetic lineages of virus. N. Engl. J. Med. 355: 909-919.

Bausch DG, Towner JS, Dowell SF, Kaducu F, Lukwiya M, Sanchez A, Nichol ST, Ksiazek TG, Rollin PE (2007). Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. J. Infect. Dis. 196 (2):S142-147.

Beer B, Kurth R (1999). Characteristics of Filoviridae: Marburg and Ebola Viruses. Naturwissenschaften 86: 8–17.

Bermejo M, Rodríguez-Teijeiro JD, Illera G, Barroso A, Vilà C, Walsh PD. (2006). Ebola outbreak killed 5000 gorillas. Science 314(5805):1564.

Boesch C (1994). Chimpanzees-red colobus monkeys: a predator-prey system. Anim. Behav. 47: 1135-1148.

Brauburger K, Hume AJ, Mühlberger E, Olejnik J (2012). Forty-Five Years of Marburg Virus Research. Viruses 4: 1878-1927.

Breman JG, Johnson KM, van der Groen G, Robbins CB, Szczeniowski MV, Ruti K, Webb PA, Meier F, Heymann DL (1999). A Search for

Ebola Virus in Animals in the Democratic Republic of the Congo and Cameroon: Ecologic, Virologic, and Serologic Surveys, 1979-1980. J. Infect. Dis. 179 (1): S139-S147.
Brosset A (1990). The migrations of *Pipistrellus nathusii* in France-possible implication on the spreading of rabies. Mammalia 54: 207-212. (In French).
Bwaka MA, Bonnet MJ, Calain P, Colebunders R, De Roo A, Guimard Y, Katwiki KR, Kibadi K, Kipasa MA, Kuvula KJ, Mapanda BB, Massamba M, Mupapa KD, Muyembe-Tamfum JJ, Ndaberey E, Peters CJ, Rollin PE, Van den Enden E, Van den Enden E (1999). Ebola hemorrhagic fever in Kikwit, Democratic Republic of the Congo: clinical observations in 103 patients. J. Infect. Dis. 179: S1-S7.
Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006). Bats: important reservoir hosts of emerging viruses. Clin. Microbiol. Rev. 19: 531-545.
Camicas JL, Wilson ML, Cornet JP, Digoutte JP, Calvo MA, Adam F, Gonzalez JP (1990). Ecology of Ticks as potential vectors of Crimean-Congo hemorrhagic fever virus in Senegal: epidemiological implications. Arch. Virol. (Suppl. 1): 303-322.
Carroll SA, Towner JS, Sealy TK, McMullan LK, Khristova ML, Burt FJ, Swanepoel R, Rollin PE, Nichol ST (2013). Molecular evolution of viruses of the family Filoviridae based on 97 whole-genome sequences. J. Virol. 87(5): 2608-2616.
Changula K, Kajihara M, Mweene AS, Takada A (2014). Ebola and Marburg virus diseases in Africa: Increased risk of outbreaks in previously unaffected areas? Microbiol. Immunol. 58: 483-491.
Chevalier MS, Chung W, Smith J, Weil LM, Hughes SM, Joyner SN, Hall E, Srinath D, Ritch J, Thathiah P, Threadgill H, Cervantes D, Lakey DL, Centers for Disease Control and Prevention-CDC (2014). Ebola virus disease cluster in the United States-Dallas County, Texas, 2014. MMWR Morb. Mortal. Wkly. Rep. 63(46):1087-1088. Erratum in: MMWR Morb. Mortal. Wkly. Rep. 63(48): 1139.
Clayton BA, Wang LF, Marsh GA (2013). Henipaviruses: An Updated Review Focusing on the Pteropid Reservoir and Features of Transmission. Zoonoses Public Health 60: 69-83.
Daszak P, Cunningham AA, Hyatt AD (2000). Emerging infectious diseases of wildlife: threats to biodiversity and human health. Science 287(5452): 443-449.
Feldmann H (2006). Marburg Hemorrhagic Fever- The Forgotten Cousin Strikes. N. Engl. J. Med. 355 (9): 866-859.
Feldmann H, Klenk HD, Sanchez A (1993). Molecular biology and evolution of filovirures. Arch. Virol. Suppl. 7: 81-100.
Fisher-Hock SP, Platt GS, Neild GH, Southee T, Baskerville A, Raymond RT, Lloyd G, Simpson DI (1985). Pathophysiology of shock and haemorrhage in a fulminating viral infection (Ebola). J. Infect. Dis. 152 (5): 887-894.
Formenty P, Boesch C, Wyers M, Steiner C, Donati F, Dind F, Walker F, Le Guenno B (1999). Ebola Virus Outbreak among Wild Chimpanzees Living in a Rain Forest of Cote d'Ivoire. J. Infect. Dis.179 (1): S120–S126.
Fujita N, Miller A, Miller G, Gershman K, Gallagher N, Marano N, Hale C (2010). Imported Case of Marburg Hemorrhagic Fever-Colorado, 2008 (Reprinted from MMWR, Vol. 58, pp. 1377-1381, 2009). J. Am. Med. Assoc. 303: 413-415.
Funk S, Piot P (2014). Mapping Ebola in wild animals for better disease control. eLife 3: e04565.
Gear JS, Cassel GA, Gear AJ, Trappler B, Clausen L, Meyers AM, Kew MC, Bothwell TH, Sher R, Miller GB, Schneider J, Koornhof HJ, Gomperts ED, Isaäcson M, Gear JH (1975). Outbreak of Marburg virus disease in Johannesburg. Br. Med. J. 4: (5995): 489-493.
Georges AJ, Leroy EM, Renaut AA, Benissan CT, Nabias RJ, Ngoc MT, Obiang PI, Lepage JP, Bertherat EJ, Bénoni DD, Wickings EJ, Amblard JP, Lansoud-Soukate JM, Milleliri JM, Baize S, Georges-Courbot MC (1999). Ebola hemorrhagic fever outbreaks in Gabon, 1994-1997: epidemiologic and Health control issues. J. Infect. Dis. 179: S65-S75.
Germain M (1978). Collection of mammals and arthropods during the epidemic of haemorrhagic fever in Zaire. In: Pattyn SR, ed. Ebola virus haemorrhagic fever. Amsterdam: Elsevier/Netherland Biomedical. pp.185-189.
Gire SK, Goba A, Andersen KG, Sealfon RSG, Park DJ, Kanneh L, Jalloh S, Momoh M, Fullah M, Dudas G, Wohl S, Moses LM, Yozwiak NL, Winnicki S, Matranga CB, Malboeuf CM, Qu J, Gladden AD, Schaffner SF, Yang X, Jiang PP, Nekoui M, Colubri A, Coomber MR, Fonnie M, Moigboi A, Gbakie M, Kamara FK, Tucker V, Konuwa E, Saffa S, Sellu J, Jalloh AA, Kovoma A, Koninga J, Mustapha I, Kargbo K, Foday M, Yillah M, Kanneh F, Robert W, Massally JL, Chapman SB, Bochicchio J, Murphy C, Nusbaum C, Young S, Birren BW, Grant DS, Scheiffelin JS, Lander ES, Happi C, Gevao SM, Gnirke A, Rambaut A, Garry RF, Khan SH, Sabeti PC (2014). Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science 345 (6202): 1369-1372.
Gonzalez JP, Gouilh MA, Reynes JM, Leroy E (2008). Bat-Borne Viral Diseases. Human health and forests. A Global Overview of Issues, Practices and Policy. Edited by Carol J. Pierce Colfer. pp. 161-200.
Gonzalez JP, Herbreteau V, Morvan J, Leroy EM (2005). Ebola virus circulation in Africa: a balance between clinical expression and epidemiological silence. Bull. Soc. Pathol. Exot. 98: 210-217.
Gonzalez JP, Pourrut X, Leroy E, 2007. *Ebolavirus* and other filoviruses. In Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and cross species transmission. JE Child, JS Mackenzie and JA Richt eds.pp. Spring verlag berlin Heidelberg. 315: 363-388.
Grard G, Biek R, Tamfum JJ, Fair J, Wolfe N, Formenty P, Paweska J, Leroy E (2011). Emergence of divergent Zaire ebola virus strains in Democratic Republic of the Congo in 2007 and 2008. J. Infect. Dis. 204 (3):S776-784.
Groseth A, Feldmann H, Strong JE. (2007). The ecology of Ebola virus. Trends Microbiol. 15 (9): 408-416.
Hayman DTS, Emmerich P, Yu M, Wang LF, Suu-Ire R, Fooks AR, Cunningham AA, Wood JLN (2010). Long term survival of an urban fruit bat seropositive for Ebola and Lagos bat viruses. PLoS ONE 5:e11978.
Hayman DTS, Meng Y, Gary C, Lin-Fa W, Suu-Ire R, Wood JLN, Cunningham AA (2012). Ebola Virus Antibodies in Fruit Bats, Ghana, West Africa. Emerg. Infect. Dis.18 (7): 1207-1209.
Huijbregts B, De Wachter P, Obiang, LSN, Akou ME (2003). Ebola and the decline of gorilla Gorilla gorilla and chimpanzee *Pan troglodytes* populations in Minkebe Forest, north-eastern Gabon. Oryx 37:437-443.
Ithete, NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, Drosten C, Drexler JF, Preiser W (2013). Close Relative of Human Middle East Respiratory Syndrome Coronavirus in Bat, South Africa. Emerg. Infect. Dis. 19 (10): 1697-1699.
Jahrling PB, Geisbert TW, Dalgard DW, Johnson ED, Ksiazek TG, Hall WC, Peters CJ (1990). Preliminary report: isolation of Ebola virus from monkeys imported to USA. Lancet 335 (8688): 502-505.
Johnson KM (1978). Ebola haemorrhagic fever in Zaire, 1976. Bull WHO 56: 271-293.
Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P (2008). Global trends in emerging infectious diseases. Nature 451(7181): 990-993.
Kenyon RH, Niklasson B, Jahrling PB, Geisbert T, Svensson L, Frydén A, Bengtsson M, Foberg U, Peters CJ (1994). Virologic investigation of a case of suspected haemorrhagic fever. Res. Virol. 145(6): 397-406.
Kiley MP, Bowen ET, Eddy GA, Isaacson M, Johnson KM, McCormick JB, Murphy FA, Pattyn SR, Peters D, Prozesky OW, Regnery RL, Simpson DI, Slenczka W, Sureau P, van der Groen G, Webb PA, Wulff H (1982). Filoviridae: a taxonomic home for Marburg and Ebola viruses? Intervirology 18 (1-2): 24-32.
Kuhn JH, Andersen KG, Baize S, Bào Y, Bavari S, Berthet N, Blinkova O, Brister JR, Clawson AN, Fair J, Gabriel M, Garry RF, Gire SK, Goba A, Gonzalez JP, Günther S, Happi CT, Jahrling PB, Kapetshi J, Kobinger G, Kugelman JR, Leroy EM, Maganga GD, Mbala PK, Moses LM, Muyembe-Tamfum JJ, N'Faly M, Nichol ST, Omilabu SA, Palacios G, Park DJ, Paweska JT, Radoshitzky SR, Rossi CA, Sabeti PC, Schieffelin JS, Schoepp RJ, Sealfon R, Swanepoel R, Towner JS, Wada J, Wauquier N, Yozwiak NL, Formenty P (2014). Nomenclature-and database-compatible names for the two Ebola virus variants that emerged in Guinea and the Democratic Republic of the Congo in 2014. Viruses 6(11):4760-4799.
Kuhn JH, Bao Y, Bavari S, Becker S, Bradfute S, Brister JR, Bukreyev

AA, Caì Y, Chandran K, Davey RA, Dolnik O, Dye JM, Enterlein S, Gonzalez JP, Formenty P, Freiberg AN, Hensley LE, Honko AN, Ignatyev GM, Jahrling PB, Johnson KM, Klenk HD, Kobinger G, Lackemeyer MG, Leroy EM, Lever MS, Lofts LL, Mühlberger E, Netesov SV, Olinger GG, Palacios G, Patterson JL, Paweska JT, Pitt L, Radoshitzky SR, Ryabchikova EI, Saphire EO, Shestopalov AM, Smither SJ, Sullivan NJ, Swanepoel R, Takada A, Towner JS, van der Groen G, Volchkov VE, Wahl-Jensen V, Warren TK, Warfield KL, Weidmann M, Nichol ST (2013). Virus nomenclature below the species level: A standardized nomenclature for filovirus strains and variants rescued from cDNA. Arch. Virol. Virus nomenclature below the species level: a standardized nomenclature for filovirus strains and variants rescued from cDNA.159(5):1229-37.

Kuhn JH, Becker S, Ebihara H, Geisbert TW, Johnson KM, Kawaoka Y, Lipkin WI, Negredo AI, Netesov SV, Nichol ST, Palacios G, Peters CJ, Tenorio A, Volchkov VE, Jahrling PB (2010). Proposal for a revised taxonomy of the family Filoviridae: Classification, names of taxa and viruses, and virus abbreviations. Arch. Virol. 155 (12): 2083-2103.

Kuno G (2001). Persistence of arboviruses and antiviral antibodies in vertebrate hosts: its occurrence and impacts. Rev. Med. Virol. 11: 165-190.

Kunz C, Hofmann H, Aspöck H (1968). Propagation of "Marburg-virus" (vervet monkey disease agent) in *Aedes aegypti*, Zentralbl. Bakteriol. Parasitol. Orig. 208: 347-349.

Kunz TH (1982). Ecology of bats. New York, NY: Plenum Press.

Kuzmin IV, Niezgoda M, Franka R, Agwanda B, Markotter W, Breiman RF, Shieh WJ, Zaki SR, Rupprecht CE (2010). Marburg Virus in Fruit Bat, Kenya. Emerg. Infect. Dis. 16: 352–354.

Lahm SA, Kombila M, Swanepoel R, Barnes RF (2007). Morbidity and mortality of wild animals in relation to outbreaks of Ebola haemorrhagic fever in Gabon, 1994–2003. Trans. R. Soc. Trop. Med. Hyg. 101(1): 64-78.

Lauber C, Gorbalenya AE (2012). Genetics-Based Classification of Filoviruses Calls for Expanded Sampling of Genomic Sequences. Viruses 4: 1425-1437.

Le Guenno B, Formenty P, Boesch C (1999). Ebola virus outbreaks in Ivory Coast and Liberia, 1994-1995, in: H.-D. Klenk (Ed.), Current Topics in Microbiol. and Immunol. Marburg and Ebola viruses. Springer-Verlag, Berlin. 235: 77-84.

Le Guenno B, Formenty P, Wyers M, Gounon P, Walker F, Boesch C (1995). Isolation and partial characterization of a new strain of Ebola. Lancet 345: 1271-1274.

Leirs H, Mills JN, Krebs JW, Childs JE, Akaibe D, Woollen N, Ludwig G, Peters CJ, Ksiazek TG (1999). Search for the Ebola virus reservoir in Kikwit, Democratic Republic of the Congo: Reflections on a vertebrate collection. J. Infect. Dis. 179 (Suppl. 1): S155-S163.

Leroy EM, Kumulungui B, Pourrut X, Rouquet P (2005). Fruit bats as reservoirs of Ebola virus. Nature 438: 575-576.

Leroy EM, Pourrut X, Gonzalez JP (2006). Bats, reserves of the Ebola virus : The mystery is dissipated. Med. Sci. 22(1): 78-79.

Leroy EM, Rouquet P, Formenty P, Souquière S, Kilbourne A, Froment JM, Bermejo M, Smit S, Karesh W, Swanepoel R, Zaki SR, Rollin PE (2004b). Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science 303 (5658) : 387-390.

Leroy EM, Telfer P, Kumulungui B, Yaba P, Rouquet P, Roques P, Gonzalez JP, Ksiazek TG, Rollin PE, Nerrienet E (2004a). A serological survey of Ebola virus infection in central African nonhuman primates. J. Infect. Dis. 190 (11):1895-1899.

Luby SP (2013). The pandemic potential of Nipah virus. Antivir. Res. 100: 38-43.

Luis AD, Hayman DT, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JR, Mills JN, Timonin ME, Willis CK, Cunningham AA, Fooks AR, Rupprecht CE, Wood JL, Webb CT (2013). A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? Proc. Biol. Sci. B 280(1756): 20122753.

Maganga GD, Kapetshi J, Berthet N, Kebela Ilunga B, Kabange F, Mbala Kingebeni P, Mondonge V, Muyembe JJ, Bertherat E, Briand S, Cabore J, Epelboin A, Formenty P, Kobinger G, González-Angulo L, Labouba I, Manuguerra JC, Okwo-Bele JM, Dye C, Leroy EM. (2014). Ebola virus disease in the Democratic Republic of Congo. N. Engl. J. Med. 371(22):2083-2091.

Martines RB, Ng DL, Greer PW, Rollin PE, Zaki SR. (2015). Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. J. Pathol. 235(2):153-174.

Martini GA (1969). Marburg agent disease in man, Trans. R. Soc. Trop. Med. Hyg. 63: 295-302.

Martini GA, Schmidt HA (1968). Spermatogenic transmission of the 'Marburg virus' (causes of 'Marburg simian disease'). *Klin Wochenschr.* 46: 398-400.

Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Alhakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, Daszak P, Al Rabeeah AA, Lipkin WI (2013). Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. Emerg. Infect. Dis. 19(11):1819-1823.

Messenger SL, Rupprecht CE, Smith JS (2003). Bats, emerging virus infections, and the rabies paradigm, In: T. H. Kunz and M. B. Fenton (ed.), Bat ecology. University of Chicago Press, Chicago, Ill: 622-679.

Mills JN (2006). Biodiversity loss and emerging infectious disease: an example from the rodent borne hemorrhagic fevers. Biodiversity 7: 9-17.

Monath TP (1999). Ecology of Marburg and Ebola viruses: Speculations and directions for future research. J. Infect. Dis. 179: S127–S138.

Morens DM, Fauci AS (2013). Emerging Infectious Diseases: Threats to Human Health and Global Stability. PLoS Pathog. 9(7): e1003467.

Morvan JM, Deubel V, Gounon P, Nakoune E, Barriere P, Murri S, Perpète O, Selekon B, Coudrier D, Gautier-Hion A, Colyn M, Volehkov V (1999). Identification of Ebola virus sequences present as RNA or DNA in organs of terrestrial small mammals of the Central African Republic. Microbes Infect. 1(14): 1193–201.

Negredo A, Palacios G, Vázquez-Morón S, González F, Dopazo H, Molero F, Juste J, Quetglas J, Savji N, de la Cruz Martínez M, Herrera JE, Pizarro M, Hutchison SK, Echevarría JE, Lipkin WI, Tenorio A (2011). Discovery of an Ebolavirus-Like Filovirus in Europe. PLoS Pathog. 7(10): e1002304.

Nkoghé D, Formenty P, Nnégué S, Mvé MT, Hypolite I, Léonard P, Leroy E (2004). Comité International de Coordination Technique et Scientifique. Practical guidelines for the management of Ebola infected patients in the field. Med. Trop. 64 (2): 199-204. [article in French].

O'Shea TJ, Cryan PM, Cunningham AA, Fooks AR, Hayman DTS, Luis AD, Peel AJ, Plowright RK, Wood JLN (2014). Bat Flight and Zoonotic Viruses. Emerg. Infect. Dis. 20 (5): 741-745.

Ogawa H, Miyamoto H, Nakayama E, Yoshida R, Nakamura I, Sawa H, Ishii A, Thomas Y, Nakagawa E, Matsuno K, Kajihara M, Maruyama J, Nao N, Muramatsu M, Kuroda M, Simulundu E, Changula K, Hang'ombe B, Namangala B, Nambota A, Katampi J, Igarashi M, Ito K, Feldmann H, Sugimoto C, Moonga L, Mweene A, Takada A. (2015). Seroepidemiological Prevalence of Multiple Species of Filoviruses in Fruit Bats (*Eidolon helvum*) Migrating in Africa. J. Infect. Dis. pii: jiv063.

Olival KJ, Hayman DTS (2014). Filoviruses in Bats: Current Knowledge and Future Directions. Viruses 6: 1759-1788.

Pan Y, Zhang W, Cui L, Hua X, Wang M, Zeng Q (2014). Reston virus in domestic pigs in China. Arch. Virol. 159(5): 1129-1132.

Parra JM, Salmerón OJ, Velasco M (2014). The first case of Ebola virus disease acquired outside Africa. N. Engl. J. Med. 371 (25): 2439-2440.

Peters CJ, Johnson ED, Jahrling PB (1992). Filoviruses. In Emerging viruses. Edited by M. S. S. Oxford: Oxford University Press, pp. 159-175.

Peterson AT, Bower JT, Mills JN (2004). Ecological and Geographic Distribution of Filovirus Disease. Emerg. Infect. Dis. 10 (1): 40-47.

Pigott DM, Golding N, Mylne A, Huang Z, Henry AJ, Weiss DJ, Brady OJ, Kraemer MU, Smith DL, Moyes CL, Bhatt S, Gething PW, Horby PW, Bogoch II, Brownstein JS, Mekaru SR, Tatem AJ, Khan K, Hay SI (2014). Mapping the zoonotic niche of Ebola virus disease in Africa. Elife 8(3):e04395.

Pourrut X (2007). Implication des chauves-souris dans les épidémies de filovirus en Afrique : modalités de contamination de l'homme et des grands singes. Thèse de Doctorat Université de la Méditerranée IX, Marseille II. 234 pp.

Pourrut X, Delicat A, Rollin PE, Ksiazek TG, Gonzalez JP, Leroy EM (2007). Spatial and Temporal Patterns of *Zaire ebolavirus* Antibody

Prevalence in the Possible Reservoir Bat Species. J. Infect. Dis. 196: S176-183.
Pourrut X, Kumulungui B, Wittmann T, Moussavou G, Délicat A, Yaba P, Nkoghe D, Gonzalez JP, Leroy EM (2005). The natural history of Ebola virus in Africa. Microbes Infect. Inst. Pasteur. 7:1005-1014.
Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, Leroy EM (2009). Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus.* BMC Infect. Dis. 9:159.
Pretorius MJ, Oelofsen MS, Smith MS, Van Der Ryst E (1997). Rift Valley Fever virus: A seroepidemiologic study of small terrestrial vertebrates in South Africa. Am. J. Trop. Med. Hyg. 57 (6): 693-698.
Rec WE (1999). Marburg fever, Democratic Republic of the Congo. Wkly. Epidemiol. Rec. 74:145.
Rep MMW (2005). Outbreak of Marburg virus hemorrhagic fever-Angola, October 1,2004-March 29, 2005. Morb. Mortal. Wkly. Rep. 54:308-309.
Richter HV, Cumming GS (2008). First application of satellite telemetry to track African straw-coloured fruit bat migration. J. Zool. (Lond). 275: 172-176.
Rodhain F (1998). La notion de réservoir en arbovirologie. Bull. Soc. Pathol. Exot. 91 (4): 279-282.
Rollin PE, Williams RJ, Bressler DS, Pearson S, Cottingham M, Pucak G, Sanchez A, Trappier SG, Peters RL, Greer PW, Zaki S, Demarcus T, Hendricks K, Kelley M, Simpson D, Geisbert TW, Jahrling PB, Peters CJ, Ksiazek TG (1999). Ebola (subtype Reston) virus among quarantined nonhuman primates recently imported from the Philippines to the United States. J. Infect. Dis.179: S108-S114.
Rosevear DR (1965). Bats of West Africa. Trustees of the British Museum (Natural History) London. 418 pp.
Rougeron V, Feldmann H, Grard G, Becker S, Leroy EM (2015). Ebola and Marburg haemorrhagic fever. J. Clin. Virol. 64:111-119.
Rouquet P, Froment JM, Bermejo M, Kilbourne A, Karesh W, Reed P, Kumulungui B, Yaba P, Délicat A, Rollin PE, Leroy EM (2005). Wild animal mortality monitoring and human Ebola outbreaks, Gabon and Republic of Congo, 2001-2003. Emerg. Infect. Dis. 11(2): 283-290.
Saéz AM, Weiss S, Nowak K, Lapeyre V, Zimmermann F, Düx A, Kuhl HS, Kaba M, Regnaut S, Merkel K, Sachse A, Thiesen U, Villányi L, Boesch C, Dabrowski PW, Radoni A, Nitsche A, Leendertz SAJ, Petterson S, Becker S, Krähling V, Couacy-Hymann E, Akoua-Koffi C, Weber N, Schaade L, Fahr J, Borchert M, Gogarten JF, Calvignac-Spencer S, Leendertz FH (2015). Investigating the zoonotic origin of the West African Ebola epidemic. EMBO Mol. Med. 7(1):17-23.
Sanderson IT (1940). The mammals of the North Cameroons forest area. Being the results of the Percy Sladen expedition to the Mamfe Division of the British Cameroons. Trans. Zool. Soc. London 24: 623-725.
Schmaljohn C, Hjelle B (1997). Hantaviruses: a global disease problem. Emerg. Infect. Dis. 3:95-104.
Siegert R, Shu HL, Slenczka HL, Peters D, Müller G (1968).The aetiology of an unknown human infection transmitted by monkeys (preliminary communication). Ger. Med. Mon. 13(1): 1-2.
Smith CEG, Simpson DIH, Bowen ETW (1967). Fatal human disease from vervet monkeys. Lancet II: 1119-1121.
Smith DH, Isaacson M, Johnson KM, Bagshawe A, Siongok T, Keruga WK (1982). Marburgvirus disease in Kenya. Lancet 1(8276):816-820.
Smith DIH (1978). Ebola haemorrhagic fever in Sudan, 1976. Bull. WHO 56:247-270.
Swanepoel R, Leman PA, Burt FJ, Zachariades NA, Braack LE, Ksiazek TG, Rollin PE, Zaki SR, Peters CJ (1996). Experimental inoculation of plants and animals with Ebola virus. Emerging Infectious Diseases. 2(4):321-325.
Taylor DJ, Leach RW, Bruenn J (2010). Filoviruses are ancient and integrated into mammalian genomes. BMC Evol. Biol. 10:193.
Timen A, Koopmans MPG, Vossen A, van Doornum GJJ, Gunther S, van den Berkmortel F, Verduin KM, Dittrich S, Emmerich P, Osterhaus AD, van Dissel JT, Coutinho RA (2009). Response to Imported Case of Marburg Hemorrhagic Fever, the Netherlands. Emerg. Infect. Dis. 15:1171-1175.
Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, Swanepoel R, Paddock CD, Balinandi S, Khristova ML, Formenty PB, Albarino CG, Miller DM, Reed ZD, Kayiwa JT, Mills JN, Cannon DL, Greer PW, Byaruhanga E, Farnon EC, Atimnedi P, Okware S, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST, Rollin PE **(**2009). Isolation of Genetically Diverse Marburg Viruses from Egyptian Fruit Bats. PLoS Pathog. 5(7):e1000536.
Towner JS, Khristova ML, Sealy TK, Vincent MJ, Erickson BR, Bawiec DA, Hartman AL, Comer JA, Zaki SR, Ströher U, Gomes da Silva F, del Castillo F, Rollin PE, Ksiazek TG, Nichol ST (2006). Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola. J. Virol. 80 (13): 6497-6516.
Towner JS, Pourrut X, Albarino CG, Nkogue CN, Bird BH, Grard G, Ksiazek TG, Gonzalez JP, Nichol ST, Leroy EM (2007). Marburg virus infection detected in a common African bat. PLoS ONE 2(8): e764.
Towner JS, Sealy TK, Khristova ML, Albariño CG, Conlan S, Reeder SA, Quan PL, Lipkin WI, Downing R, Tappero JW, Okware S, Lutwama J, Bakamutumaho B, Kayiwa J, Comer JA, Rollin PE, Ksiazek TG, Nichol ST (2008). Newly discovered ebola virus associated with hemorrhagic fever outbreak in Uganda. PLoS Pathog. 4(11):e1000212.
Turell MJ, Bressler DS, Rossi CA (1996). Lack of virus replication in arthropods after intrathoracic inoculation of Ebola Reston virus. Am. J. Trop. Med. Hyg. 55: 89-90.
Van der Poel WH, Lina PH, Kramps JA (2006). Public health awareness of emerging zoonotic viruses of bats: a European perspective. Vector Borne Zoonotic Dis. 6 (4): 315-24.
Vogel G (2014). Infectious Disease. Genomes reveal start of Ebola outbreak. Science 345 (6200): 989-990.
Walker EP (1999). Mammals of the World. (2 volumes). (Sixth Edition). Volume I- Baltimore and London: The Johns Hopkins University Press, 836 pp.
Walsh PD, Abernethy KA, Bermejo M, Beyers R, De Wachter P, Akou ME, Huijbregts B, Mambounga DI, Toham AK, Kilbourn AM, Lahm SA, Latour S, Maisels F, Mbina C, Mihindou Y, Obiang SN, Effa EN, Starkey MP, Telfer P, Thibault M, Tutin CE, White LJ, Wilkie DS (2003). Catastrophic ape decline in western equatorial Africa. Nature 422 (6932): 611-614.
Warrell MJ, Warrell DA (2004). Rabies and other lyssavirus diseases. Lancet 363:959-969.
Wauquier N, Bangura J, Moses L, Khan SH, Coomber M, Lungay V, Gbakie M, Sesay MSK, Gassama IAK , Massally JLB, Gbakima A, Squire J, Lamin M, Kanneh L, Yillah M, Kargbo K, Roberts W, Vandi M, Kargbo D, Vincent T, Jambai A, Guttieri M, Fair J, Souris M, Gonzalez JP (2015). Understanding the Emergence of Ebola Virus Disease in Sierra Leone: Stalking the Virus in the Threatening Wake of Emergence. Plos Current Outbreaks. doi: 10.1371/currents.outbreaks.9a6530ab7bb9096b34143230ab01cdef.
Weingartl HM, Nfon C, Kobinger G (2013). Review of Ebola virus infections in domestic animals. Dev. Biol. (Basel).135:211-218.
WHO Ebola Response Team (2014). Ebola Virus Disease in West Africa-The First 9 Months of the Epidemic and Forward Projections. N. Engl. J. Med. 1-15.
Woolhouse ME, Haydon DT, Antia R (2005). Emerging pathogens: the epidemiology and evolution of species jumps. Trends Ecol. Evol. 20: 238-244.
World Health Organization (WHO) (1978). Ebola haemorrhagic fever in Zaire, 1976. Bull. World Health Organ. 56:271–293.
World Health Organization (WHO) (1992). Viral haemorrhagic fever in imported monkeys. Wkly. Epidemiol. Rec. 67:142-143.
World Health Organization/International Study Team (1978). Ebola haemorrhagic fever in Sudan, 1976. Report of a WHO/International Study Team. Bull World Health Organ. 56:247-270.

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Occurrence of *Campylobacter* species in beef cattle and local chickens and their antibiotic profiling in Ibadan, Oyo State, Nigeria

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Food animals like cattle and poultry are often regarded as reservoirs for *Campylobacter* infections in human. This study investigated the occurrence of *Campylobacter coli* in cattle and local chickens and their antibiotic susceptibility to commonly used antibiotics in Ibadan, Oyo State, Nigeria. A total of 250 samples comprising 100 rectal swabs, 100 gall bladder contents from cattle and 50 cloacal swabs from local chickens that were apparently healthy, were subjected to standard microbiological identification and antibiotic susceptibility tests. Overall, 51 (20.4%) *C. coli* were isolated including 34/100 (34%) from rectal swabs, 12/100 (12%) from gall bladders and 5/50 (10%) from the cloaca. All the isolated *C. coli* displayed multiple antibiotic resistances to between 4 and 10 of the antibiotics tested showing up to 40 different resistance patterns. The cattle *C. coli* displayed a high frequency of resistance to erythromycin and ciprofloxacin, while all the chicken isolates were resistant to erythromycin, the drug of choice for the treatment of the *Campylobacter* infections in Nigeria. This investigation carried out in apparently healthy animals identified cattle and local chickens as potential reservoir hosts for *C. coli* infection in the study area.

Key words: Campylobacter coli, local chickens, multiple antibiotic resistance, Ibadan.

INTRODUCTION

Campylobacter is a Gram-negative, spiral shaped, obligate microaerophilic, motile bacterium, having up to 23 species documented in the NCBI taxonomy division (Moolhueijzen et al., 2009). Morphologically, they are helical or curved shaped with long spiral forms which resemble spirochaetes superficially. *Campylobacter* species are motile by means of flagella which are usually single at one or both poles (Barrow and Feltham, 1993; Moolhueijzen et al., 2009). Campylobacteriosis, an important bacteria zoonoses is caused by species from the Genus *Campylobacter* (Tambur et al., 2013). The Thermophilic species such as *Campylobacter jejuni, C. coli, C laris,* and *C. upsaliensi* are the most common causative agents of human diseases (Tambur et al., 2013).

Campylobacter species, particularly *C. jejuni and C. coli* are commonly traced to foodborne illnesses in the United States and worldwide (CDC, 2013; Scallan et al.,

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2011). For instance, they accounted for approximately 35% of laboratory confirmed foodborne illnesses within the FoodNet surveillance areas in the United States in 2012 (CDC, 2013). *C. jejuni* and *C. coli* were mostly reported during the period with *C. jejuni* responsible for 80-90% of human infections (CDC, 2013; Nachamkin and Blaser, 2000). Based also on European Food safety Authority report for 2010, there were 212064 confirmed cases of campylobacteriosis, making it to be the most reported zoonosis in European Union during the period (Anonymous, 2010). *Campylobacter* was reported to be mainly distributed in poultry; however cattle, pigs, sheep and pet animals were also acknowledged as the possible sources of Campylobacter infection (Anonymous, 2010; 2012a). The prevalence of the bacteria in retail fresh broilers meat in EU region varied between 3.1 to 58.8% depending on the member of State as from 2006 (Anonymous, 2010; 2012). Most Campylobacteriosis in New-Zealand around 2005 were attributable to *C. jejuni* and only around 10% were associated with *C. coli* (Moore et al., 2005).

These organisms are known to colonize different hosts including human and other animals with varying degrees of virulence (Fouts et al., 2005). Although chickens have been its most frequently identified reservoir for human infection, *Campylobacter* species have been isolated from other sources such as the faeces of healthy cattle (Humphrey et al., 2007; Baserisehalehi et al., 2007; Mohammed et al., 2009; Salihu et al., 2009). Cattle strains can infect poultry suggesting cattle as possible reservoir for poultry infections (Ziprin et al., 2003). The organism may also be carried asymptomatically by a wide range of animals and excreted into the environment in faeces (EPIDAT, 2005; Moore et al., 2005). Humans can thus be infected by several non-human hosts through consumption of contaminated water, or from food animals and their products (Rodrigues et al., 2001; Kapperud et al., 2003; Stanley and Jones, 2003; Teunis et al., 2005). However, contamination during food processing has been identified as the most important means of *Campylobacter* infections and the characteristics of the organism such as motility, ability to adhere to intestinal mucosa, capability to invade enterocytes as well as toxin production have been associated with its pathogenicity (Datta et al., 2003; Dasti et al., 2010).

Campylobacteriosis is usually a self-limiting disease and thus do not usually require antimicrobial treatment (Wieczorek et al., 2012). In some cases however such as septiceamic form of the disease characterized by severe and prolonged enteritis, in immune-compromised or young patients, antimicrobial therapy may be required; and in such cases, macrolides (erythromycin) and quinolones/ fluoroquinolones (ciprofloxacin, nalidixic acids) are usually the drugs of choice (Skirrow and Blaser, 2000; Engberg et al., 2001; Wieczorek et al., 2012).

According to Lehtopolku (2011), multidrug resistance in *Campylobacter* is associated with resistance to the drug of choice like the macrolides and fluoroquinolones for the treatment of the life threatening infections, whereas those resistant to three or more group of antimicrobial agents apart from the macrolides could be referred to as multiple drug resistant organisms (Lehtopolku, 2011). The multidrug resistant *Campylobacter* is often associated with the presence of the *CmeABC* multidrug efflux pump (Lehtopolku, 2011). There have been various reports of multidrug resistance *Campylobacter* species in different parts of the world. For instance, 2.2% incidence of multidrug resistance *Campylobacter* species was reported between 1989 and 1993 in North India (Prasad et al., 1994). From the same region there was an increase to 30.6% among *C. jejuni* and *C. coli* in 2002 and 90% for 2008 (Jain et al., 2005; Chen et al., 2010). In China, 76.8% incidence of multidrug resistant *C. coli* was reported, and the strains showed 19 different multiple antimicrobial patterns (Qin et al., 2011).

In the Northern Nigeria, Salihu et al. (2009) documented the prevalence of 65.1% for *C. jejuni,* 23.0% for *C. coli,* 7.9% for *C. laris,* 3.2% for *C. hyointestinalis* and 0.8% for *C. fetus.* This paper reports the occurrence of *Campylobacter species* in beef cattle and local chicken and their antibiotic sensitivity in Ibadan, Oyo State, Southwestern Nigeria.

MATERIALS AND METHODS

Sample collection/location

A total of 250 samples comprising of 100 rectal swabs and 100 swab samples of gall bladder contents from slaughtered cattle in Municipal abattoir Bodija, Ibadan Oyo State and 50 cloacal swabs from local chickens at Abadina Community, University of Ibadan and from Igbo oloyin area of Ibadan were collected. Ibadan, the biggest city in the South Western Nigeria, hosts the biggest cattle market and abattoir in the region. Cattle and local chickens were sampled by insertion of a sterile swab (Global swab®) into the rectums and cloaca, respectively. Each swab was placed in Amies charcoal transport medium (Oxoid CM 0425®) and transported to laboratory within 5 hours in ice packs. The laboratory analysis of the sample was carried out at the Nigerian Institute of Science Laboratory Technology (NISLT), Ibadan.

Bacteriological processing

The samples were analysed for the thermotolerant *Campylobacter* species as earlier described (Skirrow and Benjamin, 1980; Georges-Courbot et al., 1986; Karmali et al., 1986; Barrow and Feltham, 1993). The cattle rectal swabs, gall bladder contents and chicken cloacal swabs were inoculated in duplicates onto modified charcoal cefoperazone deoxycholate agar (MCCDA Oxoid CM0739®, and incubated microaerobically at 25°C (to allow for the growth of *Campylobacter fetus)* and 42°C respectively, for 48 h. The microaerophilic environment of 5% O_2, 10% CO_2, and 85% N_2 was produced using Campygen sachet (Oxoid CN0025A®) inside an anaerobic jar. The suspected *Campylobacter* colonies were Gram - stained and subjected to further biochemical tests: catalase and oxidase tests, urease production, H_2S production, nalidixic acid and cephalothin sensitivity tests, growth at 42°C and hippurate hydrolysis (Gerhardt et al., 1984). Each isolate was stored at -80°C in a peptone broth with 15% glycerol for further analysis.

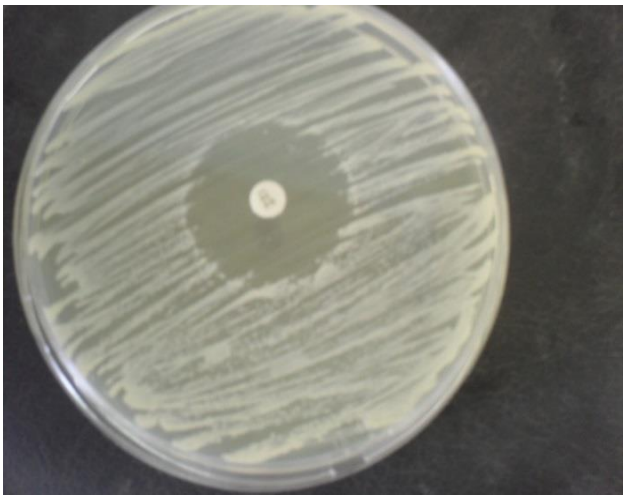

Figure 1. *Campylobacter* susceptibility to cephalothin.

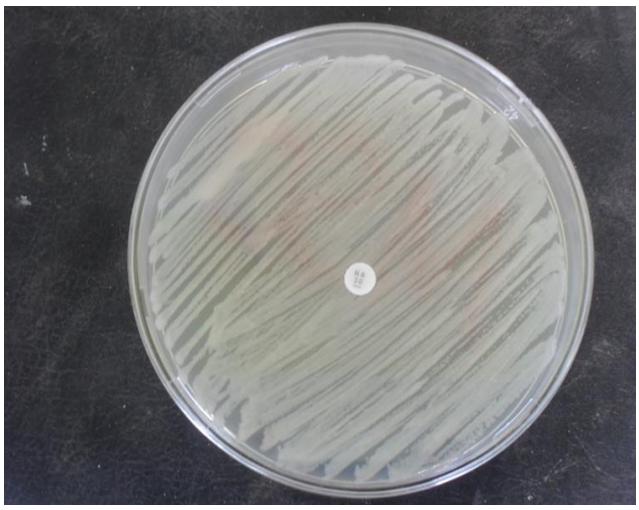

Figure 2.*Campylobacter* resistance to Nalidixic acid.

Hippurate hydrolysis

The test was carried out to differentiate between *C. coli* and *C. jejuni*. A large loopful of suspected *Campylobacter* colonies were scraped from the MCCDA plates and mixed with hippurate solution to form a very cloudy suspension, the tube was incubated in water bath at 37^{o}C for 2 h. Subsequently, 0.2 mL of ninhydrin reagent was added without shaking the tubes and incubated at 37°C for 10 min. Formation of a deep purple colour due to glycine formation from hippurate hydrolysis indicated presence of *C. jejuni*, while absence of colour formation indicated presence of *C. coli* (Gerhardt et al., 1984).

Antimicrobial susceptibility testing

The *in-vitro* antibiotics sensitivity of the *Campylobacter* isolates was carried out by agar disc diffusion test (Matsen and Barry, 1974) using disc of amoxicillin (25 µg), ofloxacin (5 µg), streptomycin (10 µg), chloramphenicol (30µg), ceftriazone (30 µg), gentamycin (10 µg), pefloxacin (5 µg), cotrimoxazole (25 µg), ciprofloxacin (10 µg), erythromycin (5 µg) on Mueller- Hinton agar (Oxoid®) at 37°C for 24 h under microaerophilic atmosphere. The results were interpreted according to the standard guideline by Clinical and Laboratory Standards Institute (CLSI, 2008).

RESULTS

Bacterial processing

All the plates incubated at 25°C for possible isolation of *Campylobacter fetus* showed no growth. The positive plates of local chicken cloacal swabs (1 from Abadina and 4 from Igbo oloyin) and cattle rectal swabs/ gall bladders incubated at 42°C showed the characteristic small, grey, butyrous, moist, flat and spreading colonies. The isolates were Gram-negative and curved rods.

Biochemically, isolates were oxidase- and catalase-positive. Isolates were motile and H_2S- negative. All the isolates produced negative reactions for hippurate hydrolysis and suggestive of *C. coli*. All the isolates were susceptible to 30 µg cephalothin (Figure 1) and resistant to 30 µg nalidixic acid (Figure 2).

Occurrence of *Campybacter*

A total of 51 (20.4%) *C. coli* were isolated from the 250 samples examined comprising of 100 rectal swabs and 100 from gall bladders from cattle, and 50 from cloacal swabs from local chickens. From the cattle rectal samples, 34/100 (34%) yielded *C. coli*, whereas 12/100 (12%) occurrences were recorded for the gall bladder samples. Cloacal swabs were 5/50 (10%) positive from apparently healthy chickens.

A total of 63% of *C. coli* from cattle were susceptible to ofloxacin followed by ceftriazone (36%). However, there were high resistance of 84.8 and 82.6% for ciprofloxacin and erythromycin, respectively (Table 1). The organisms that produced 17 to 27 mm clearing zones for 10 µg of ciprofloxacin and 18 to 22 mm for 5 µg of erythromycin were adjudged susceptible, whereas all the isolates considered resistant did not produce any clearing zones.

Likewise, from the local chickens there was a 100% susceptibility to ofloxacin followed by 60% susceptibility to ciprofloxacin, but the 5 isolates from the local chicken cloacal were 100% resistant to amoxicillin, streptomycin, chloramphenicol, ceftriazone, gentamycin and erythromycin (Table 2).

The 40 different multiple antibiotics resistance patterns exhibited by the isolates from cattle and chickens are shown in Table 3. In cattle, there were five different resistance patterns for 10 antimicrobial agents, 3 patterns for 9, 5 patterns for 8, 17 patterns for 7, 10 patterns for 6, 2 patterns for 5 and 1 pattern for 4 antimicrobial agents.

For the local chickens; there was 1 pattern for resistance to 9 antimicrobial agents, 3 patterns for 7, and 1 pattern for 6.

Table 1. Antimicrobial Susceptibilities of Cattle isolates.

Antibiotics	Number of resistant isolates (%)
Amoxycillin	32/46 (69.6)
Ofloxacin	17/46 (37.0)
Streptomycin	37/46 (80.4)
Chloramphenicol	31/46 (67.4)
Ceftriazone	29/46 (63.0)
Gentamycin	36/46 (78.0)
Pefloxacin	35/46 (76.1)
Cotrimoxazole	33/46 (71.7)
Ciprofloxacin	39/46 (84.8)
Erythromycin	38/46 (82.6)

Table 2. Antimicrobial Susceptibilities of local chicken isolates.

Antibiotics	Number of resistant isolates (%)
Amoxycillin	5/5 (100)
Ofloxacin	0/5 (0)
Streptomycin	5/5 (100)
Chloramphenicol	5/5 (100)
Ceftriazone	5/5 (100)
Gentamycin	5/5 (100)
Pefloxacin	4/5 (80)
Cotrimoxazole	3/5 (60)
Ciprofloxacin	2/5 (40)
Erythromycin	5/5 (100)

DISCUSSION

Phenotypic characteristics of *C. coli* isolated during this study agree with the description given by Debruyne et al. (2009) namely growth at 42°C, catalase positive, hippurate negative, nalidixic acid resistant and susceptible to cephalothin. In this investigation no *C. jejuni* was isolated and the occurrence of 34% *C. coli* from cattle rectal samples in the current study is higher than 25% *C. coli* reported by Mohammed et al. (2009) from rectum of cattle in Sokoto State, a Northern region of Nigeria. Earlier studies demonstrated that most cases of cattle *Campylobacter* species infections were associated with *C. jejuni* than *C. coli* (Inglis et al., 2004). Stanley et al. (1998) reported 89% occurrence of *Campylobacter* from small intestines of cattle. The isolation rate (12%) of C. *coli* from cattle gall bladders in this study was lower than 47% reported in a previous study by Muz et al. (1992) and 35.6% Acik and Cetinkaya (2005) outside, Nigeria. The *C. coli* recovered from gall bladders and faecal samples agreed with those Acik and Cetinkaya (2005) who earlier documented the organism to be a commensal in the various organs of healthy cattle. This study shows that gall bladders of cattle harbor *Campylobacter* and may result in contamination of carcass during unhygienic slaughtering and subsequent transmission to human beings. Wild birds, domestic and companion animals are known as reservoirs for *Campylobacter* species, and they shed the organisms in faeces contaminating the environment (Akitoye et al., 2002). Occurrence of 10% *C. coli* from apparently healthy local chickens is noteworthy. In Nigeria, local chickens are found within households, hence, they are important economically and constitute a source of transmission of *Campylobacter* organisms to human. One report showed that strains isolated from human and chickens were phenotypically and genotypically correlated, confirming that chickens are an important source of human campylobacteriosis in developing countries including Nigeria (Adegbola et al., 1990).

The antibiotic sensitivity test revealed low susceptibility by these *C. coli* to most of the 10 antibiotics studied. The cattle *C. coli* isolates exhibited low susceptibility to ciprofloxacin and erythromycin, while all the chicken *C. coli* were resistant to amoxicillin, streptomycin, chloramphenicol, ceftriazone, gentamycin and erythromycin; those resistant *Campylobacter species* to erythromycin and ciprofloxacin conform to the definition of multidrug resistance (Lehtopolku, 2011) because they are resistant to the drug of choice for treating Campylobacter infections when need be. The observed 18 to 22 mm clearing zone for the erythromycin susceptible *C. coli* in this study is comparable to those of Gaudreau et al. (2007) where susceptible *C. coli* had a clearing zones of ≥ 15 mm at erythromycin MIC ≤ 4 mg/L. The ciprofloxacin susceptibility in this study was based on clearing zones of 17 to 27 mm which is slightly different from ≥ 25 mm zone of clearing around 5 µg ciprofloxacin as reported by the same author (Gaudreau et al., 2007).

A better susceptibility was however observed for ofloxacin both in cattle and chicken isolates. The antibiotics resistance in this study is similar to that of Sammarco et al. (2010) who found *Campylobacter coli* isolated from chicken and beef meat to be resistant to most antibiotics tested in Italy. Chatre et al. (2010) in France also documented an upward trend in resistance of *Campylobacter* species isolated from cattle to commonly used antibiotics notably quinolones, aminoglycosides and penicillins. The antibiotics resistance exhibited by *C. coli* observed in this investigation also agrees with observations from other parts of the world, as observed from food and water sources as well as from clinical samples reported in Europe (Moore et al., 2001; San'enz et al., 2000); Canada (Gaudreau and Gilbert, 1998), and the United States (CDC, 2000).

Fluoroquinolone, like ciprofloxacin and erythromycin are often regarded as the drugs of choice for treatment of patient with severe campylobacteriosis, while tetracycline, doxycycline, and chloramphenicol are sometimes listed

Table 3. Antibiotic resistance patterns of Campylobacter coli isolated from Cattle and local chickens.

Serial number	Resistant pattern	Number of antibiotics	Frequency	Animal source
1	Amx, Ofl, Str, Chl, Cef, Gen, Pef, Cot, Cpx, Ery	10	5	Cattle
2	Amx, Str, Chl, Cef, Gen, Pef, Cot, Cpx, Ery	9	3	Cattle
3	Amx, Str, Chl, Cef, Gen, Cot, Cpx, Ery	8	1	Cattle
4	Ofl, Str, Cef, Gen, Pef, Cot, Cpx, Ery	8	1	Cattle
5	Str, Chl, Cef, Gen, Pef, Cot, Cpx, Ery	8	2	Cattle
6	Amx, Chl, Cef, Gen, Pef, Cot, Cpx, Ery.	8	1	Cattle
7	Amx, Str, Chl, Gen, Pef, Cot, Cpx, Ery	8	1	Cattle
8	Amx, Chl, Cef, Pef, Cot, Cpx, Ery	7	1	Cattle
9	Str, Chl, Cef, Pef, Cot, Cpx, Ery	7	1	Cattle
10	Amx, Str, Chl, Gen, Pef, Cpx, Ery	7	1	Cattle
11	Amx, Str, Gen, Pef, Cot, Cpx, Ery	7	2	Cattle
12	Ofl, Str, Chl, Gen, Pef, Cpx, Ery	7	1	Cattle
13	Str, Chl, Cef, Gen, Pef, Cpx, Ery	7	1	Cattle
14	Amx, Str, Chl, Pef, Cot, Cpx, Ery	7	1	Cattle
15	Amx, Str, Chl, Gen, Cot, Cpx, Ery.	7	1	Cattle
16	Amx, Ofl, Chl, Cef, Gen, Cot, Cpx	7	1	Cattle
17	Amx, Chl, Gen, Pef, Cot, Cpx, Ery	7	1	Cattle
18	Ofl, Str, Cef, Gen, Pef, Cpx, Ery	7	1	Cattle
19	Amx, Str, Chl, Gen, Pef, Cot, Ery	7	1	Cattle
20	Amx, Str, Cef, Pef, Cot, Cpx, Ery	7	1	Cattle
21	Amx, Ofl, Str, Cef, Gen, Cot, Ery.	7	1	Cattle
22	Amx, Str, Chl, Cef, Gen, Cot, Ery	7	1	Cattle
23	Ofl, Str, Gen, Pef, Cot, Cpx, Ery	7	1	Cattle
24	Ofl, Str, Chl, Gen, Cot, Ery	6	1	Cattle
25	Amx, Str, Cef, Gen, Cpx, Ery	6	1	Cattle
26	Cef, Gen, Pef, Cot, Cpx, Ery	6	1	Cattle
27	Amx, Str, Gen, Pef, Cot, Ery	6	1	Cattle
28	Amx, Str, Cef, Pef, Cpx, Ery	6	1	Cattle
29	Amx, Ofl, Chl, Cef, Pef, Cpx	6	1	Cattle
30	Ofl, Str, Chl, Gen, Pef, Cpx	6	1	Cattle
31	Amx, Str, Cef, Gen, Cot, Ery	6	1	Cattle
32	Amx, Chl, Cef, Pef, Cpx, Ery	6	1	Cattle
33	Amx, Ery, Ofl, Chl, Cot, Cpx	6	1	Cattle
34	Amx, Chl, Cef, Gen, Ery	5	1	Cattle
35	Amx, Chl, Cef, Gen, Ery	5	1	Cattle
36	Amx, Str, Cpx, Ery	4	1	Cattle
37	Amx, Str, Chl, Cef, Gen, Pef, Cot, Cpx, Ery	9	1	Chicken
38	Amx, Str, Chl, Cef, Gen, Cot, Ery	7	2	Chicken
39	Amx, Str, Cef, Gen, Pef, Cpx, Ery	7	1	Chicken
40	Amx, Str, Chl, Cef, Gen, Ery	6	1	Chicken

as alternative drugs (Luangtongkum et al., 2009; Jong et al., 2009). The low susceptibility of the C. coli to ciprofloxacin calls for concern. However, such a phenol-menon suggests the misuse/abuse of the drug by most livestock farmers and dealers without proper prescription by professionals in Nigeria (Unpublished data). Prudent use of the commonly used antibiotic tested in this study, particularly those drugs of choice for treatment of Campylobacter infection is recommended.

Conflict of interest

The author(s) have not declared any conflict of interests.

REFERENCES

Açik M, Çetinkaya B (2005). The heterogeneity of *Campylobacter jejuni* and *Campylobacter coli* strains isolated from healthy cattle. Lett. Appl. Microbiol. 41:397-403.

Adegbola RA, Alabi SA, Akinkuade FO, Coker AO, Odugbemi T (1990). Correlation between human and animal bio-serogroups of *Campylobacter* isolates in Nigeria. J. Trop. Med. Hyg. 93:280-283.

Akitoye OC, Raphael D, Isokpehi DR, Bolaji N, Thomas, Kehinde OA, Larry OC (2002). Human Campylobacteriosis in Developing Countries. Emerg. Infect. Dis. 8 (3):237-243.

Anonymous (2012).The Europian Union Summary report on trends and

sources of zoonoses, zoonotic agent and foodborne agent and food borne outbreaks in 2010. EFSA journal 10, 2597. bacteria, 3rd edition. Cambridge. UK. Cambridge University Press. 331.
Anonymous (2010). Analysis of the baseline survey on prevalence of Campylobacter in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU 2008 part A: *Campylobacter* and *Salmonella* prevalence estimates. EFSA J. 8:1503.
Baserisehalehi M, Bahadur N, Kapadnis BP (2007). Isolation and characterization of *Campylobacter* species from domestic animals and poultry in South Iran. Pak. J. Biol. Sci. 10 (9):1519-1524.
CDC (2013). Incidence and trends of Infection with pathogens transmitted commonly through food- foodborne disease active surveillance network, 10 US sites 1996-2012 MMWR Morb. Mortal. Weekly Rep. 62:283-287.
CDC (2000). Center for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) (1999) annual report. [Online.]http://www.cdc.gov/narms.
Chatre P, Haenni M, Meunier D, Botrel M, Calavas D, Madec J (2010). Prevalence and Antimicrobial Resistance of Campylobacter jejuni and Campylobacter coli Isolated from Cattle between 2002 and 2006 in France. J. Food Prot. 73(5):825–831.
CLSI (2008). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard- 3rd Edn. CLSI document M31-A3, 1-99. Cliniucal and Laboratory Standards Institute, 940 West Valley Road, Wayne, Pennsylvania, USA. 2008; 28:8.
Dasti JI, Tareem AM, Lugart R, Zautner AE, Gross U (2010). *Campylobacter jejuni*: A brief overview on pathogenicity-associated factors and disease- mediated mechanism. Int. J. Med. Microbiol. 300:205-211.
Datta S, Niwa H, Itoh K (2003). Prevalence of II pathogenic genes of Campylobacter jejuni by PCR in strains isolated from from humans, poultry meat and broiler and bovine faeces. J. Med. Microbiol. 52: 345-348.
Debruyne L, On SL, Brandt ED, Vandamme P (2009). Novel *Campylobacter*lari-like bacteria from humans and molluscs: description of *Campylobacterpeloridis* sp. nov., *Campylobacterlari* subsp. *concheus* subsp. nov.and*Campylobacterlari* subsp. *lari* subsp. nov. Int. J. Syst. Evol. Microbiol. 59:1126–1132.
Engberg J, Aeestrup FM, Taylor DE, Garner-Smidt P, Nachamkin I (2001). Quinolone and macrolide resistance in Campylobacter jejuni and C coli: resistance and trends inhuman isolates. Emerg. Infect. Dis. 7:24-34.
EPIDAT (2005).Incidence of selected infectious diseases in the Czech Republic, years 1995-2004.Zprávy CEM 14:54-55.
Fouts DE, Mongoden EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Perker CT, Daughty SC (2005). Major structural differences and Novel potential virulence mechanisms from the genomes of multiple Campylobacter species. Plos Biol. 3(1):15.
Gaudreau C, Girouard Y, Ringuette L, Tsimiklis C (2007). Comparison of disk diffusion and agar dilution mrthod for erythromycin and ciprofloxacin susceptibility testing of Campylobacter coli and for tetracycline susceptibility testing of Campylobacter jejuni subsp jejuni. Antimicrob. Agents Chemother. 51:1524-1526.
Gaudreau C, Gilbert H (1998). Antimicrobial resistance of clinical strains of *Campylobacter jejuni*subsp. *jejuni*isolated from 1985 to 1997 in Quebec, Canada. Antimicrob. Agents Chemother. 42:2106-2108.
Georges-Courbot MC, Baya C, Beraud AM, Meunier DMY, Georges AJ, (1986). Distribution and Serotypes of *Campylobacter jejuni* and *Campylobacter coli* in Enteric *Campylobacter* Strains Isolated from Children in the Central African Republic. J. Clin. Microbiol. 23(3): 592-594.
Gerhardt P, Murray RGE, Costilow, RN, Nester EW, Wood WA, Kneg NR, Philips GB (1984). Manual of methods for General Bacteriology.American Society for Microbiology Washington DC 20006. 524pp.
Humphrey TS, O'Brien S, Madsen M (2007). *Campylobacter* in Zoonotic pathogens: a food production perspective. Int. J Food Microbiol. 117:234-257.
Inglis GD, Kalischuk LD, Busz HW (2004). Chronic shedding of *Campylobacter* species in beef cattle. J. Appl. Microbiol. 97:410-420.
Jong de AR, Bywater P, Butty E, Deroove K, Godinho U, Klein H, Marion S, Simjee K, Smets V, Thomas M, Valle´ Wheadon A (2009). A pan-European survey of antimicrobial susceptibility towards human-use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food-producing animals. J. Antimicrob. Chemother. 63:733-744.
Kapperud G, Espeland G, Wahl E, Walde A, Herikstad H, Gustavsen S, Tveit I, Natas O, Bevanger L, Digranes A (2003). Factors associatedwith increased and decreased risk of *Campylobacter* infection: a prospective case-control study in Norway. Am. J. Epidemiol. 158:234-242.
Karmali MA, Simor AE, Roscoe M, Flemming PC, Smith SS, Lane J (1986). Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. J. Clin. Microbiol. 23:456-459.
Lehtopolku M (2011). Antimicrobial Resistance in *Campylobacter jejuni* and *Campylobacter coli*. Department of Internal Medicine and Medical Microbiology and Immunology, University of Turku, Turku, Finland and the Antimicrobial Resistance unit, National Institute for Health and Welfare(Former National Public Health Institute, KT4, Yurku, Finland ISBN 978-951-4718-8.
Luangtongkum T, Jeon B, Han J, Plummer P, Logue C M, Zhang Q (2009). Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. Future Microbiol. 4:189-200.
Matsen JM Barry AL (1974). Manual of Clinical Microbiology 2nd edition.American Society for Microbiology, Washington, DC. pp. 418-427.
Mohammed DS, Junaidu AU, Oboegbulem SI, Egwu GO, Mogaji AA, Lawal M, Hassan Y (2009). Isolation and prevalence of *Campylobacter* species in Sokoto State, Nigeria. Vet. Ital. 45(4):501-505.
Moolhueijzen PM, Lew-Tabor AE, Wlodek BM, Aguero FG, Comerci DJ, Ugalde RA, Sanchez DO, Appels R, Bellgard M (2009). Genomic analysis of Campylobacter fetus subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiol. 9:86-97.
Moore J E, Corcoran D, Dooley JS, Fanning S, Lucey B, Matsuda M, McDowell DA, Megraud F, Millar BC, O'Mahony R,'Riordan L.O, Rourke MO, Rao JR, Rooney P J, Sails A, Whyte P (2005). *Campylobacter*. Vet. Res. 36:351-382.
Moore JE, Crowe M, Heaney N, Crothers E (2001). Antibiotic resistance in *Campylobacter* spp. isolated from human faeces (1980–2000) and foods (1997–2000) in Northern Ireland: an update. J. Antimicrob. Chemother. 48:455-457.
Muz A, Ozcan C, Gurcay M, Angin M (1992). Investigation on aerobic, anaerobic and microaerophilic bacteria in the gall-bladders of sheep and cattle slaughtered in meat and fish organization in Elazig. F. U. Sag BilDerg 6:98-104.
Nachamkin I, Blaser MJ (2000). *Campylobacter*, 2nd edition. Washington: American Society for Microbiology;
Prasad KN, Mathur SK, Dhole TN, Ayyagari A (1994). Antimicrobial susceptibility and plasmid analysis of Campylobacter jejuni isolated from diarrhoel patients and healthy chickens in Northern India. J. Diarrhoeal Dis. Res. 12(4):270-273.
Qin SS, Wu CM, Wing Y, Jeon B, Shen ZQ, Wing Y, Zhang Q, Shen JZ (2011). Antimicrobial resistance in Campylobacter coli isolated from pigs in in two province of China. Int. J. Food Microbiol. 146(1): 94-98.
Rodrigues L C, Roberts JA , Cumberland P, Sockett PN, Wheeler J, Cowden JM, Wheeler JG, Sethi D, Wall PG, Cumberland P, Tompkins DS, Hudson MJ, Roberts JA, Roderick PJ (2001). The study of infectious intestinal disease in England: riskfactors for cases of infectious intestinal disease with *Campylobacter jejuni* infection. Epidemiol. Infect. 127:185-193.
Sa´enz Y, Zarazaga M, Lantero M, Gastan˜ares MJ, Baquero F, Torres C (2000). Antibiotic resistance in *Campylobacter* strains isolated fromanimals, foods, and humans in Spain in 1997–1998. Antimicrob. Agents Chemother. 44:267-271.
Salihu MD, Abdulkadar JU, Oboegbulem SI, Egwu GO, Magaji AA, Lawal M, Hassan Y (2009). Isolation and prevalence of Campylobacter species in Cattle from Sokoto State, Nigeria. Vet. Ital. 45(4): 501-505.
Sammarco ML, Ripabelli G, Fanelli I, Grasso MG, Tamburro M (2010).

Prevalence and biomolecular characterization of campylobacter spp. isolated from retail meat. J. Food Prot. 73(4):720-728.
Scallan E, Hoestra RM, Angulo FJ, Tauxe RV, Widdowson MA, Ray SL, Jeffrey L, Jones JL, Griffin PM (2011). Foodborne illness acquired in the United States major pathogen. Emerg. Infect. Dis. 17:7-15.
Skirrow MB, Benjamin J (1980). "1001" *Campylobacters*: cultural characteristics of intestinal *Campylobacters* from man and animals. J. Hyg. Camb. 85:427-442.
Skirrow MB, Blaser MJ (2000).Clinical aspects of *Campylobacter* infection. In: I. Nachamkin and Blaser. M. J. (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C. pp. 69-88.
Stanley K, Jones K (2003).Cattle and sheep farms as reservoirs of *Campylobacter*. J. Appl. Microbiol. 94(Suppl):104-113.
Stanley KN, Wallance JS, Currie JE, Diggle PJ Jones K (1998).The seasonal variation of thermophilic*Campylobacters* in beef cattle, dairy cattle and calves. J. Appl. Microbiol. 85:472-480.
Tambur Z, Miljkovic-Selimovic B, Doder R, Kulisic Z(2013). Susceptibility of *Campylobacter jejuni* and *Campylobacter coli* isolated from animals and humans to tetracycline. Afr. J. Poult. Farming 1(2):37-40.
Teunis P, Van den Brandhof W, Nauta M, Wagenaar J, Van den Kerkhof H, Van Pelt W (2005). A reconsideration of the Campylobacterdose-response relation. Epidemiol. Infect. 133:583-592.
Wieczorek K, Szewczy KR, Osek J (2012). Prevalence, antimicrobial resistance and molecular characterization of Campylobacter jejuni and Campylobacter coli isolated from retail raw meat in Poland. Vet. Med. 57(6):293-299.
Ziprin RL, Sheffield CL, Hume ME, Drinnon DLJ, Harvey RB (2003). Ceacal colonization of chicks by bovine derived strains of Campylobacter. Avian Dis. 47:1429-1433.

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Full Length Research Paper

Effect of temperature of storage on the composition and microbiological quality of raw milk

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The aim of the study was to evaluate the quality of refrigerated raw milk from dairy farms located in Southwestern state of Goiás during the rainy and dry seasons. Fresh milk samples were collected from bulk tank and stored for 0, 24, 48 and 72 h for the evaluation of psychrotrophic microorganisms counts, titratable acidity, chemical composition, somatic cell count (SCC) and total bacterial count (TBC). In the rainy season, the average temperature of the refrigerated raw milk samples was 17.4, 6.0, 6.1 and 5.3°C and in the dry period, the average temperature was 9.2, 2.4, 3.8 and 1.4°C for 0, 24, 48 and 72 h of storage, respectively. The physicochemical characteristics of refrigerated milk were consistent with the maximum limits established by Brazilian legislation after storage for 72 h in expansion tanks; however, in the dry period, refrigerated milk should not remain stored for more than 24 h due to the high TBC values. The results of the microbiological analyses revealed failures in the cleaning of equipment and utensils used for milking, demonstrating need for greater hygiene in the collection and maintenance of refrigerated milk at the production source.

Key words: Storage time, mastitis, refrigerated milk, hygiene.

INTRODUCTION

Aspects such as storage of refrigerated raw milk for up to 48 h at temperatures <7°C were established by Normative Instruction 51 (Brazil, 2002), in addition to somatic cell count (SCC) <750,000 SC/mL and total bacterial count (TBC) <750,000 CFU/mL, which remained until 2011. With current Normative Instruction 62, these limits have changed, for somatic cell count are allowed (SCC) maximum of 600,000 SC/mL and total bacterial count (TBC) of 600,000 CFU/mL.

In practice, it has been observed that after the implementation of granelizada milk collection, there is storage for more than 48 h at the source of production because the expansion tanks allow milk storage of the various milking, thereby reducing transportation costs.

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However, maintenance of refrigerated raw milk in expansion tanks for extended periods, provide development of psychrotrophic and proteolytic psychrotrophic count which was found in the study of Santos et al. (2009).

The storage of milk refrigerated in bulk tank is maintained at temperatures <7°C for up to 48 h but does not have enhancing effect on the milk quality since according to Guinot-Thomas et al. (1995), the changes in milk composition (decrease in pH and casein content) caused by the action of proteinases originating from psychrotrophic microorganisms begin when the microbial count reaches between 10^6 and 10^7 CFU / mL, which occurs after four days of storage at 4°C.

The objective of this study was to evaluate the quality of refrigerated raw milk stored for up to 72 h on expansion tanks from farms located in Southwestern state of Goiás during the rainy and dry seasons.

MATERIALS AND METHODS

Sampling

Refrigerated raw milk samples from bulk tank were collected during the rainy and dry seasons directly from expansion tanks installed on farms, whose owners were milk suppliers of a Dairy Industry located in Southwest Goiás, making up a total of 28 samples per season. Seven milk producing farms already established in the bulk collection program of this dairy industry were selected, so that there would not be withdrawals during the trial period. A sample was collected from each producer.

The milk remained stored for a period of 72 h in expansion tank. Milking was performed once a day, and in five farms, cows were milked by hand and in two other farms, milking was mechanical. The farms used had average production of 100 L of milk/day, had crossbred herd with access to Brachiaria pasture in the rainy season and in the dry season, in addition to grazing, animals received sugarcane to complement the diet. In all farms, animals received vaccinations provided by the board of health protection of the State of Goiás.

Milk samples were collected with 0, 24, 48 and 72 h of storage, which were characterized as mixed refrigerated raw milk (reassembly milk) from four milkings. The collection of refrigerated raw milk samples was performed after cooling for at least two hours in the expansion tank. Milk temperature (°C) was measured at the sampling time using a thermometer.

Laboratory analyses

To assess SCC and chemical composition, samples were collected in flasks containing preservative Bronopol®, and for TBC, flasks containing azidiol were used. To assess the psychrotrophic count and titratable acidity, milk samples were aseptically collected using stainless steel collector and stored in amber flasks (± 250 mL). Soon after collection, samples were placed in cool isothermal box containing ice and sent for analysis.

Chemical composition was determined using MilkoScan 4000 equipment and results were expressed as percentage. SCC was held in Fossomatic 5000 Basic equipment and the result was expressed in SC/mL. TBC was analyzed using the BactoScan FC equipment and results were expressed as CFU/ml.

For psychrotrophic count, milk samples were diluted by aseptically pipetting up 25 mL in Erlenmeyer type flask containing 225 mL of 0.1% peptone water (dilution 10^{-1}). From this dilution, decimal dilutions were prepared up to 10^{-6}. About 1 ml of dilutions was added to sterile Petri dishes in duplicate and 15 ml to 17 ml of standard agar for counting were added, molten and cooled to 45°C and homogenized (APHA, 2001). After agar solidification at room temperature, the plates were incubated at 7°C/10 days (Marshall, 1992). Counts were performed in colony counter on plates containing between 25 to 250 colonies. To calculate the number of colony forming units (CFU)/mL, number of colonies on each plate was multiplied by inverse of the inoculated dilution.

For proteolytic psychrotrophic counts, decimal dilutions were prepared as described for the psychrotrophic count. Subsequently, 1 mL of dilutions was added to sterile Petri dishes and 15 mL to 17 mL of milk agar (standard agar plus 10% skimmed milk powder reconstituted to 10%) freshly prepared, melted and cooled to 45°C. Plates were incubated at 21°C/72 h (Marshall, 1992). When plates were read, chemical precipitant was used (10% acetic acid) to identify the presence of proteolysis. Colonies with transparent halo were counted and the number of CFU/mL was calculated by multiplying the number of colonies on each plate by the inverse of the dilution.

For *Pseudomonas* spp. count, decimal dilutions were prepared as described for the psychrotrophic count. After the completion of dilutions, 0.1 ml was added to sterile Petri dishes adding 15 to 17 ml of *Pseudomonas* Agar Base plus 5 ml of glycerol, samples were inoculated in culture medium, spread with Drigalski loop, and immediately incubated at 28°C for 48 h. At the end of this period, reading and interpretation were held (King et al., 1954). The results were expressed as CFU/ml. Titratable acidity was performed according to Brasil (2006) and the results were expressed as grams (g) of lactic acid/100 ml.

Data were submitted to analysis of variance with the following factors being analyzed: season (rainy or dry) and storage time (0, 24, 48 and 72 hours) in a completely randomized design and 2 x 4 factorial arrangement. Bacterial count was analyzed by means of regression models using the Microsoft Excel software. To meet the assumptions of the analysis of variance, variables were transformed using the natural logarithm (ln x) resulting in: ln (psychrotrophic), ln (proteolytic psychrotrophic), ln (Pseudomonas +1), ln (protein), ln (SCC), ln (TBC). These transformations were performed in order to reduce the range of data. Statistical analyses were performed using the SISVAR Software (Ferreira, 2003).

RESULTS AND DISCUSSION

Table 1 show average temperature of refrigerated raw milk samples at the time of sampling, during the rainy season and dry. The temperature of fresh milk samples (zero hour) was higher in the early hours of storage in both the rainy season and in the dry season because at the collection time, the milk had not been completely cooled. According to Fagundes et al. (2004), at the second hour after milking, temperature should be 4°C. According to Brasil (2011), the storage temperature of refrigerated raw milk at the production source should be below 7°C within three hours after milking.

The average titratable acidity results (Table 2) significantly differed between seasons. In the dry season, titratable acidity was higher than in the rainy season; being 0.16; 0.17; 0.17 and 0.17 for 0; 24; 48 and 72 h, respectively, however, during the storage time of up to 72 h at the production source, no significant difference in the

Table 1. Temperature of refrigerated raw milk samples at the time of sampling, during the rainy and dry season.

	Storage (hours)	Rainy (°C)	Dry (°C)
Temperature	zero	17.4	9.2
	24	6.0	2.4
	48	6.1	3.8
	72	5.3	1.4

Table 2. Mean titratable acidity and chemical composition values of refrigerated milk stored for up to 72 h at the production source during the rainy and dry seasons.

Season	Storage (hours)	Titratable acidity	Fat (%)	Protein (%)	Lactose (%)	EST (%)	ESD (%)
Rainy	0	0.16	3.66	3.25	4.50	12.41	8.75
	24	0.16	3.65	3.26	4.46	12.38	8.73
	48	0.15	3.69	3.26	4.45	12.40	8.71
	72	0.16	3.70	3.25	4.42	12.38	8.68
Mean		0.16b	3.68b	3.26a	4.46a	12.39b	8.72b
Dry	0	0.16	4.27	3.30	4.55	13.10	8.83
	24	0.17	3.99	3.35	4.67	13.00	9.01
	48	0.17	3.96	3.35	4.66	12.95	9.00
	72	0.17	3.99	3.29	4.58	12.85	8.86
Mean		0.17a	4.05a	3.32a	4.62a	12.98a	8.93a

Same letters in the same column do not differ statistically from each other at 5% significance. Titratable acidity results are expressed in grams of lactic acid/100 ml of milk.

titratable acidity was found. The results found are within limits established by Brazilian legislation from 0.14 to 0.18 g of lactic acid/100 mL of milk (Brasil, 2011).

During storage of milk for 72 h, no significant changes in the titratable acidity results were found. The collection of reassembly milk resulted in samples with different characteristics every 24 h, but with no changes in the titratable acidity results. Although the titratable acidity results had not differed between seasons, the higher acidity observed in the dry season may be related to the higher bacterial count observed in this period, which resulted in a significant increase in titratable acidity.

There was a significant difference in the fat content (Table 2) of refrigerated milk samples according to the season. During the dry season, the fat content was higher than in the rainy season; however, no significant differences were observed during the storage period. The average fat content obtained in the rainy season may be related to the diet offered to animals, which consisted of *Brachiaria*.

To maintain stable rumen function and prevent depression in content milk fat, NRC (2001) recommends minimum 25% dietary fiber, measured as detergent fiber neutral, with 75% of the total diet being supplied by forage.

The mean protein values (Table 2) observed in this study were higher than those obtained by Noro et al. (2006) (3.10% in the rainy period and 3.17% in the dry season) and Gonzalez et al. (2004) (2.98% in the rainy period and 2.87% in the dry season).

There was no significant difference for the lactose content (Table 2) during storage and between seasons. The average lactose results observed in this study were similar to those obtained by Noro et al. (2006), who reported mean values of 4.46 (rainy season) and 4.55% (dry season).

The average EST results (Table 2) during storage of refrigerated milk for up to 72 h did not differ significantly. The mean EST values were significantly different between seasons, with higher results in the dry season. The mean EST values of this study were higher than the results obtained by Gonzalez et al. (2004), with mean of 12.08% for the rainy period and 12.04% for the dry period and Martins et al. (2006), with mean of 11.41% for the rainy period and 11.24% for the dry season; however, these researchers observed greater EST during the rainy season.

The mean ESD values (Table 2) significantly differed between seasons. There was no significant difference during storage in refrigeration tanks for up to 72 h at the production source. The ESD values of the present study were higher than the results obtained by Gonzalez et al. (2004), which were 8.39 (rainy season) and 8.42% (dry season) and by Martins et al. (2006) who obtained 8.3 (rainy season) and 8.0% (dry season).

The mean chemical composition results of refrigerated

Table 3. Mean SCC and TBC values of refrigerated milk stored for up to 72 h at the production source during the rainy and dry seasons.

Season	Storage (hours)	SCC (CS/mL)	TBC (CFU/mL)
Rainy	0	295.857	136.143
	24	286.857	115.429
	48	305.000	237.143
	72	286.857	421.571
Mean		293.643a	227.572a
Dry	0	498.429	359.429
	24	487.857	1.966.429
	48	472.571	3.371.429
	72	498.857	1.858.286
Mean		489.429a	1.888.893a

Same letters in the same column do not statistically differ from each other at 5% significance.

raw milk samples stored for 72 h at the production source in the different seasons are in line with Brasil (2011), who found minimum fat, protein, EST and ESD contents of 3.0; 2.9, 11.4 and 8.4%, respectively.

Similar results were found by Andrade et al. (2014) where medios fat values (3.48%); protein (3.29%); EST (12.13%) and ESD (8.65%) were seen in the dry hazard. Already in the rainy hazard were found the following results for fat (3.59%); protein (3.31%); EST (12.25%) and ESD (8.66%).

Brazilian legislation for raw milk quality determines storage time of refrigerated milk of up to 48 h in the farm and recommends 24 h as maximum storage time; however, some dairies collect refrigerated milk stored for more than 48 h in expansion tanks. Longer storage time can be attributed to factors such as storage capacity that allows the storage of several milkings and reduction of freight costs due to higher milk volume collected on the farm.

As there are no studies that evaluate the characteristics of refrigerated raw milk stored for more than 48 h on the farm, comparisons used in this study refer to the different seasons. In assessing the milk quality in production systems in Southern state of Rio Grande do Sul, Zanela et al. (2006) reported that only 41.8% of milk samples were within limits established by law, and that the chemical composition standards of milk required by Brazilian law should be revised, considering regional variations.

The average SCC results (Table 3) of refrigerated milk samples did not differ between seasons and storage time; however, higher SCC values can be observed in the dry season, which agrees with Bueno et al. (2005) in the State of Goiás.

The mean SCC values obtained in this research for the rainy and dry seasons are in line with Brasil (2011), who established maximum limit of 600,000 SC/mL. These values were lower than those described by Machado et al. (2000), who evaluated the quality of milk stored in expansion tanks of some regions. These researchers obtained mean value of 641,000 SC/mL, with standard deviation of 767,000 SC/mL, and standard deviation larger than the mean value was attributed to the large variation among herds analyzed. The high SCC values in milk obtained from expansion tanks suggest loss of milk production and low SCC is indicative of good health status of the mammary gland.

Although the TBC values showed a wide variation (Table 3), the results did not differ between seasons and storage time. However, TBC values were higher in the dry season. The mean TBC values in the rainy season, regardless of storage time, were within limits established by Brazil (2011), but in the dry season, the values found from the 24 h of storage were higher than the limit of 600,000 CFU/ml of milk allowed by law. In the dry season, the mean TBC result was high, which would make milk not to be in compliance with requirements of Normative Instruction 62/2011. Similar results were found by Bozo et al. (2013) where the values of SCC and TBC were higher in the dry season.

The TBC values obtained in this study are related to the study by Silveira et al. (2000), who reported that the microbial load present in fresh milk is influenced by the season, production and handling practices on the farm, geographic location, milk temperature and distance between farm and dairy industry.

Storage of raw milk under refrigeration for long periods at the dairy farm and bulk transportation to the processing industry can increase milk TBC because according to Baruffaldi et al. (1984), the bacteriological quality of freshly milked milk are specific to each region, and the mixture of milks of various origins can compromise the quality of the final blend due to the introduction of various microbial levels.

The mean psychrotrophic count (Table 4) significantly differed between seasons, and higher count was observed in the rainy season, but the results were not significant during the storage period. The mean proteolytic psychrotrophic count (Table 4) did not differ

Table 4. Mean psychrotrophic, proteolytic psychrotrophic and *Pseudomonas* spp. count of refrigerated milk stored for up to 72 h at the production source during the rainy and dry seasons.

Season	Storage (hours)	Psychrotrophic	Proteolytic psychrotrophic	*Pseudomonas* spp.
Rainy	0	77.843	42.171	20.496
	24	594.857	66.286	48.371
	48	201.143	96.429	19.906
	72	328.857	400.571	99.344
Mean		300.675a	151.364a	47.029a
Dry	0	13.929	32.786	17.286
	24	35.314	43.929	4.286
	48	530.157	138.129	3.571
	72	500.614	53.286	1.857
Mean		270.004b	67.033a	6.750b

Same letters in the same column do not statistically differ from each other at 5% significance. Results are expressed as CFU/mL.

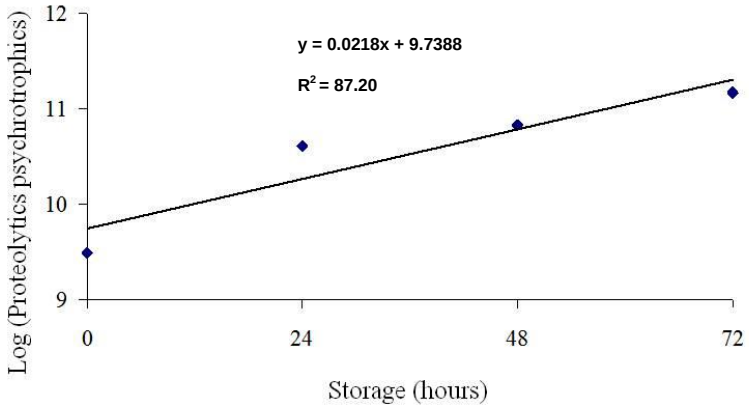

Figure 1. Proteolytic psychrotrophic count of refrigerated milk stored for up to 72 h at the production source during the rainy and dry seasons.

significantly between seasons; however, higher proteolytic psychrotrophic count was observed during the rainy season. The mean *Pseudomonas* spp. count (Table 4) significantly differed between seasons, with higher counts during the rainy season.

The results obtained in this study differ from those obtained by Santos et al. (2013), with higher temperature (4, 7 and 10°C) and storage time (24, 48, 72 and 96 h) greater the counts of psychrotrophic.

The results obtained in this study differ from those obtained by Pinto et al. (2006), who reported a variation from 2.0×10^2 to 1.0×10^7; 5.0×10^1 to 1.2×10^6 and 1.0×10^1 to 3.8×10^6 CFU/mL for psychrotrophic, proteolytic psychrotrophic and *Pseudomonas* spp. count, respectively. According to Fox (1989), psychrotrophic bacteria are apparently not significant as to proteolysis unless the population exceeds 10^6 CFU/mL. The increased proteolytic psychrotrophic bacteria count observed in this study (Table 4) can lead to increased proteolysis in milk and dairy products. According to Vidal-Martins et al. (2005), during storage of UHT milk, increased proteolysis index and apparent viscosity during storage was observed, which could be related to the presence of proteases produced by psychrotrophic bacteria in raw milk.

The psychrotrophic count of milk stored under refrigeration for up to 72 h during the rainy season was higher than the limit of 10% stipulated for this type of microorganism in milk (Brasil, 1980). Among psychrotrophic bacteria, *Pseudomonas* spp. are the predominant spoilage bacteria in refrigerated raw milk, particularly *Pseudomonas fluorescens*. According to Muir (1996), in newly milked milk, *Pseudomonas* spp., are present in about 10% of the total microbiota, but in milk kept under refrigeration, these bacteria have predominance over the other species present in both fresh and processed milk.

The proteolytic psychrotrophic count (Figure 1) resulted in increasing linear behavior (R^2 = 87.20%) during the storage time of refrigerated raw milk at the production

source in the rainy and dry seasons. The result obtained for the proteolytic psychrotrophic count allowed identifying that after milking, this group of microorganisms showed significant growth in the first 72 h of storage.

Firstly, psychrotrophic bacteria are responsible for producing thermostable proteases and lipases, causing significant damage to the dairy chain. Although, the proteolytic activity in milk may be due to enzymes originating from somatic cells (Santos et al., 2003), because according to Santos et al. (2006), milk with high SCC had higher proteolysis rate during storage.

Pedrico et al. (2009) reported that, to meet the requirements of Brazilian legislation, quality policies involving public agencies, technicians and industry should be disseminated due to the need to develop activities aimed at improving the quality of milk.

Conclusions

The average titratable acidity of milk differed significantly between seasons. There was a significant difference in the fat content according to the season, and in the dry season, the fat content was higher than in the rainy season. The mean SCC values obtained in this study for the rainy and dry seasons were within standards required by Brazilian legislation. In the dry period, refrigerated milk should not remain stored for more than 24 h due to the high TBC values. The high occurrence of psychrotrophic bacteria during the rainy season may be related to poor hygiene practices during milking. Thus, for refrigerated milk to meet the requirements of Brazilian legislation regarding TBC, measures aimed at the explanation of the milk production chain in relation to the need for producing milk with adequate sanitary quality and that does not result in public health problems should be adopted.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Andrade KD, Rangel AHN, Araújo VM, Medeiros HR, Bezerra Kc, Bezerril RF, Júnior DML (2014). Qualidade Do Leite Bovino Nas Diferentes Estações Do Ano No Estado Do Rio Grande Do Norte. R. Bras. Ci. Vet. 21(3):213-216.

American Public Health Association (APHA) (2001). Compendium of methods for the microbiological examination of foods. 4. ed. Washington: APHA, pp. 676

Baruffaldi R, Penna TCV, Machoshvili LA, Abe LE (1984). Condições higiênico-sanitárias do leite pasteurizado tipo "B" vendido na cidade de São Paulo, SP (Brasil), no período de fevereiro a agosto de 1982. Revista de Saúde Pública, São Paulo. 18(5):367-374.

BrasiL RB, Silva MAP, Carvalho TS, Cabral JF, Nicolau ES, Neves RBS (2012) . Avaliação da qualidade do leite cru em função do tipo de ordenha e das condições de transporte e armazenamento. Revista do Instituto de Laticínios Cândido Tostes. 67(389):34-42.

BRASIL (2011). Instrução Normativa nº 62, de 29 de dezembro de. Aprovar o Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, o Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, o Regulamento Técnico de Identidade e Qualidade de Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Diário Oficial da República Federativa do Brasil, Brasília, 30 de dezembro de 2011. Seção 1, p.1-24.

BRASIL (2002). Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de Origem Animal. Instrução Normativa no 51, de 18 de setembro de 2002. Aprova e Oficializa o Regulamento técnico de identidade e qualidade de leite cru refrigerado. Diário Oficial da República Federativa do Brasil 172(13-22):20 set.. Seção I.

BRASIL (1980). Ministério da Agricultura, Pecuária e Abastecimento. Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal – RIISPOA. Brasília. pp. 116.

BRASIL (2006). Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Instrução Normativa nº 68, de 12 de dezembro de 2006. Oficializa os Métodos Analíticos Oficiais Físico-Químicos, para Controle de Leite e Produtos Lácteos. Diário Oficial da União de 14/12/2006, Seção 1, Página 8 Brasília, DF.

Bozo GA, Alegro LCA, Silva LC, Santana EHW, Okano W, Silva LCC (2013) Adequação da contagem de células somáticas e da contagem bacteriana total em leite cru refrigerado aos parâmetros da legislação. Arq. Bras. Med. Vet. Zootec. 65(2):589-594.

Bueno V FF, Mesquita A J, Nicolau ES, Oliveira AN, Oliveira JP, Neves RBS, Mansur JRG, Thomaz LW (2005). Contagem celular somática: relação com a composição centesimal do leite e período do ano no Estado de Goiás. Ciência Rural, Santa Maria, 35(4):848-854.

Fagundes CM, Fischer V, Silva WP, Carbonera N, Araujo MR (2004). Presença de Pseudomonas spp em função de diferentes etapas da ordenha com distintos manejos higiênicos e no leite refrigerado. Anais do XXI Congresso Nacional de Laticínios, Juiz de Fora. pp. 290-293.

Ferreira DF (2003). Sisvar: versão 4.3 (Build 43). Lavras: Departamento de Ciências Exatas, Universidade Federal de Lavras.

Fox PF (ed.) (1989) Developments in Dairy Chemistry – 4. Functional Milk Proteins. ElServier Applied Science, Elsevier Science Publisher Ltd. London and New York.

Gonzalez HL, Fischer V, Ribeiro MER, Gomes JF, Waldyr SJR, Silva MA (2004). Avaliação da qualidade do leite na bacia leiteira de Pelotas, RS. Efeito dos meses do ano. Revista Brasileira de Zootecnia, Viçosa, 33(6):1531-1543.

Guinot-Thomas P, Ammoury M, Roux Y, Laurent F (1995). Study of proteolysis during storage of raw milk at 4°C: effect of plasmin and microbial proteinases. Int. Dairy J. 5:685-697.

King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Lammers BP, Buckmaster DR, Heinrichs AJ (1996). A simple method for the analysis of particle sizes of forage and total mixed rations. J. Dairy Sci. 79(5):922-928.

Machado PF, Pereira AR, Silva LFP, Sarriés GA (2000). Células somáticas no leite em rebanhos brasileiros. Scientia Agrícola, Piracicaba, 57(2):359-361.

Marshall RT (1992). Standard Methods for the Examination of Dairy Products. American Public Health Association, 16 ed.

Martins PRG, Silva C A, Fischer V, Ribeiro MER, Stumpf WJ, Zanela MB (2006). Produção e qualidade do leite na bacia leiteira de Pelotas-RS em diferentes meses do ano. Ciência Rural, Santa Maria, 36(1):209-214.

Muir DD (1996). The shelf-life of dairy products: factors influencing raw and fresh products. J. Soc. Dairy Technol. 49:24-32.

Noro G, Gonzalez FHD, Campos R, Durr JW (2006). Fatores ambientais que afetam a produção e a composição do leite em rebanhos assistidos por cooperativas no Rio Grande do Sul. Revista Brasileira de Zootecnia, Viçosa, 35(3):1129-1135.

Pedrico A, Castro JGD, Silva JEC, Machado LAR (2009). Aspectos higiênico-sanitários na obtenção do leite no assentamento alegre, município de araguaína, TO. Ciência Animal Brasileira, 10(2):610-617.

Pinto CLO, Martins ML, Vanetti MCD (2006). Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. Ciência e Tecnologia de Alimentos, Campinas, 26(3):645-651.

Santos AS, Pires CV, Santos JM, Sobrinho PSC (2013). Crescimento de micro-organismos psicrotróficos em leite cru refrigerado. Alim. Nutr. Braz. J. Food Nutr. Araraquara, 24(3):297-300.

Santos MV, Ma Y, Barbano DM (2003). Effect of somatic cell count on proteolysis and lipolysis in pasteurized fluid milk during shelf-life storage. J. Dairy Sci. 86:2491-2503.

Santos MV, Oliveira CAF, Lima YVR, Botaro BG (2006). Remoção de células somáticas pela microfiltração não afeta a composição e a proteólise do leite. Ciência Rural, Santa Maria, 36(5):1486-1493.

Santos PA, Silva MAP, Souza CM, Isepon JS, Oliveira AN, Nicolau ES (2009). Efeito do tempo e da temperatura de refrigeração no desenvolvimento de microrganismos psicrotróficos em leite cru refrigerado coletado na macrorregião de Goiânia – GO. Ciência Animal Brasileira 10(4):1237-1245.

Silveira IA, Carvalho EP, Teixeira D (2000). Influência de microrganismos psicrotróficos sobre a qualidade do leite cru refrigerado. Uma revisão. Higiene Alimentar, 12(55):21-27.

Vidal-Martins AMC, Salotti BM, Rossi Junior OD, Penna A LB (2005). Evolução do índice proteolítico e do comportamento reológico durante a vida de prateleira de leite UAT/UHT. Ciência e Tecnologia de Alimentos, Campinas, 25(4):698-704.

Zanela MB, Fischer V, Ribeiro MER, Stumpf WJ, Zanela C, Marques LT, Martins PRG (2006). Qualidade do leite em sistemas de produção na região Sul do Rio Grande do Sul. Pesquisa Agropecuária Brasileira, Brasília, 41(1):153-159.

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Microbiological and mycotoxicological evaluation of rice products used in human food in northeastern Brazil

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Rice consumption is part of Brazilian food culture, the per capita consumption, considering different forms is approximately of 14.9 kg of rice. The storage of rice grains in inappropriate conditions favor fungal growth and mycotoxin production. A survey was carried out to determine presence of coliforms, *Salmonella, Bacillus cereus,* fungal and mycotoxin contamination (aflatoxins, ochratoxin A and zearalenone) in 40 rice products (rice flakes and rice dough) samples traded in Terezina. Also, the ability to produce mycotoxins by *Aspergillus* and *Fusarium* isolates was shown. Regarding the microbiological standards, the results were within the established pattern. Several fungal species, especially *Aspergillus flavus* and *Penicillium citrinum*, were isolates, but the strains were not able to produce aflatoxins and citrinin, respectively. The samples commercialized in Terezina had satisfactory hygienic and sanitary conditions, and free of mycotoxins analyzed.

Key words: Rice flakes, rice dough, mycotoxins, fungi, bacteria.

INTRODUCTION

Rice production is of fundamental importance in the world, because it is considered a staple food in many countries. Brazil is among the top ten world producers with 11 million tons produced per year. Santos et al. (1994) points

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out that the Asian countries, where a considerable amount of rice is consumed daily, the problem of fungal contamination and mycotoxin in this cereal is relevant. Rice is part of the food habits of the brazilian people, which is confirmed by high consumption, considering its different forms. The average consumption of rice in Brazil is approximately 12 million tonnes (Conab, 2012).

For the great importance of their consumption, studies have reported the involvement of this cereal in outbreaks of microbiological contamination, both by fungi, such as bacteria. *Bacillus cereus* is a natural soil bacterium and can also contaminate the rice planting, remain viable in the form of spores and subsequent treatment to withstand the processing of rice (Ghelard, 2002). Other bacteria such as *Salmonella*, an important causative agent of infections have been described as contaminants in feed ingredients, such as corn, sorghum, rice bran and cottonseed meal (Jones and Richardson, 2004). The occurrence of *Salmonella* in these grains may be related to the stages of growth, harvest, storage or transport, as well as to contamination by effluents, sewage, fecal waste, where this pathogen can be incorporated into crops by irrigation system (Freitas et al., 2003).

The monitoring of fungal contamination of rice is indispensable to ensure the quality and safety of this cereal (Guimarães et al., 2010). The fungi are widely distributed in the environment, being frequently contaminants of food, especially of plant origin. Some species may invade and colonize plant tissues during all stages of production: cultivation, harvesting, drying, transport, in the processing and storage (Rodriguez-Amaya and Sabino, 2002; Galvano et al., 2005). The storage of grains such as rice, under inadequate conditions favor the growth of fungi, during development to produce secondary metabolites called mycotoxins that affect human and animal health (Tanaka et al., 2007).

For the development of fungi and their mycotoxins, they need favorable conditions, and the most important factors are: temperature, water activity and moisture content, pH, chemical composition of food, rate of oxygenation, storage period, degree of fungal contamination, physical conditions of grain or seeds, arthropods and microbial interaction (Boeing, 2003). The fungal genera *Aspergillus* and *Penicillium* are considered the main fungi contaminating grains such as rice, corn, wheat, sorghum, nuts and cotton seeds used in the formulation of foodstuffs (Rodriguez-Amaya and Sabino, 2002). Its capacity to grow at high temperatures and low water activity makes settlers of several crops (Moss, 1991).

Some species of the genus *Aspergillus* are important producers of mycotoxins, such as aflatoxins (AFs), ochratoxin (OTA) (Cast, 2003). Within the genus *Penicillium,* some species produced a variety of mycotoxins such as cyclopiazonic and penicilic acid, citreoviridin, citrinin, ochratoxin A, patulin, roquefortine and others (Pitt and Hocking, 2008). The ingestion of mycotoxins could cause various detrimental health effects by inducing different clinical signs and lesions, where these will be linked to the type of mycotoxin, dose and incubation period (Dilkin and Mallmann, 2006). The presence of fungi with the capability to produce mycotoxins in foods does not confirm the presence of these, but only the possibility of contamination. Moreover, the absence of these fungi does not ensure the food is clear of these compounds, because these toxins persist for a long time after the fungus has lost its viability (Yoshisawa, 2001).

The high frequency of consumption of rice and its by-products, as a potential source of mycotoxins there is a need for information on the microbiological quality and mycotoxin contamination in this cereal in areas of northern and northeastern Brazil. Therefore, the study aimed to evaluate the microbiological quality, the presence of fungi and mycotoxins in rice products.

MATERIALS AND METHODS

Forty samples were used (500 g each), 20 g rice flakes (marks: A, B, C and D) and 20 g of mass rice (marks: E, F, G and H) sold in different supermarkets in the City of Terezina - Piaui, Brazil. The data collection period was from January to May 2011. After collecting the samples, they were homogenized and mixed, quartered, to obtain 100 g samples for the analysis. The mycological evaluation was performed immediately, and aliquots were stored for the mycotoxin analysis.

Each sample (25 g) was diluted with 225 mL of peptone water 0.1% (wt/v). This mixture was shaken and decimally diluted (10^{-2} and 10^{-3}). Dilutions performed in peptonated water were incubated for 24 h at 37°C for the *Salmonella* presence as recommended by APHA (2005). The analysis of total and thermotolerant coliforms, as well as *Bacillus cereus* also was carried out following the methodology described by APHA (2005).

For fungi analysis, 0.1 mL aliquot of each dilution (duplicate) per sample was spread on the surface of solid media dichloran- Rose Bengal chloramphenicol-(DRBC) (Pitt and Hocking, 2007). The plates were incubated for 7 days at 25°C. All plates containing 10 - 100 CFU were counted and the results denominated in colony forming units (CFU) per gram of sample. At the last day of incubation, colonies of *Aspergillus* and *Penicillium*, after microscopic identification according to criteria proposed by Pitt and Hocking (2008), were transferred to malt extract agar (MEA) and incubated at 25°C for 7 days for subsequent species identification. For the identification of *Penicillium*, colonies were grown on Czapek yeast agar (CYA) at 5, 25 and 37°C MEA at 25°C and 25% glycerol nitrate agar (G25N) at 25°C. To identify *Aspergillus*, the cultures are grown on CYA (25 and 37°C), MEA (25°C) and Czapek yeast extract agar with 20% sucrose (CY20S) at 25°C. All the plates were incubated for 7 days. Each strain was identified according to the methods provided by Pitt (1988) and Klich and Pitt (1994). To determine producing strains of citrinin, we used the method described by Lin and Dianese (1976), if inoculating isolated *Penicillium citrinun* In Medium Coco CAM (Cocunut-Agar-Medium) and further read on cromatovisor to 366 nm.

The strains of *Aspergillus flavus*, were evaluated in their potential to produce mycotoxins using the method described by Soares and Rodriguez-Amaya (1989). *A. flavus* strains were grown on MEA plates at 25°C for 7 days, the mycelium was transferred to an Eppendorf micro-tube and 1000 µL of chloroform was added. The

Table 1. Content of coliforms, *Salmonella* spp., and *Bacillus* spp. in rice sub-products.

Sample	Average Fungi CFU/g in Log_{10}	Coliforms at 35°C in NMP/g	Coliformes at 45°C in NMP/g	Absence/ presence *Salmonella* spp/25 g	Absence/ presence *Bacillus* spp/25 g
A (n=5)	3.65	1.98	1.20	Absence	Absence
B (n=5)	4.17	2.77	0.69	Absence	Absence
C (n=5)	3.12	2.01	0.90	Absence	Absence
D (n=5)	3.39	1.62	0.69	Absence	Absence
E (n=5)	3.58	1.09	0.47	Absence	Absence
F (n=5)	3.18	1.11	0.47	Absence	Absence
G (n=5)	2.96	0.92	0.47	Absence	Absence
H (n=5)	3.16	1.09	0.47	Absence	Absence

Table 2. Percentage (%) of filamentous fungi isolated from rice sub-products intended for human consumption.

Fungal genera	No. isolates	Frequency (%)
Aspergillus e teleomorfos	47	35.6
Penicillium	39	29.5
Cladosporium	24	18.2
Fusarium	09	6.8
Mucorales	07	5.2
Curvularia	03	2.4
Chaetosartorya	02	1.5
Emiricella	01	0.8
Total	132,0	100

mixture was stirred at 4000 rpm for twenty minutes, the mycelium was removed and the extract of chloroform evaporated at environmental temperature. The residue was redissolved in 200 µL of chloroform and extracts were tested for aflatoxin by thin layer chromatography (TLC). The extracts were analyzed by chromatography on silica gel plates 60 F254, TLC aluminum plates (20 x 20 cm, thickness 250 µm, Merck, Germany). The liquid carrier was chloroform : acetone (90:10 v / v). The detection limit of the method used is 5 µ/g.

The presence of mycotoxins (aflatoxins, ochratoxin A and zearalenone) in rice product samples were analyzed follow the methodology proposed by Soares and Rodriguez-Amaya (1989). Briefly, 50 g of sample was extracted with 270 mL of methanol and 30 mL of 4% of potassium chloride, after filtering the filtrate was clarified with 30% ammonium sulfate and celite. The mixture was filtered through qualitative filter paper and 150 mL of filtrate (clarified extract) was transferred to a separatory funnel. The toxins were extracted by liquid-liquid partitions with chloroform twice. The organic phase was combined and evaporated to dryness in-route steam at 80°C. The residue was dissolved in 200 µL of benzene. For toxin identification and quantification silica gel plates 60 F254, TLC aluminum plates (20 x 20 cm, thickness 250 µm, Merck, Germany) were used. The plate was development with toluene-ethyl acetate-formic acid (60:40:0.5). For the visualization of aflatoxins B1, B2, G1 and G2 and ochratoxin A plates were placed under UV lamp 366 nm. For zearalenone the silica gel plate was sprayed with 20% aluminum chloride in 75 % ethanol and heated at 110°C for five minutes.

The results of the counts were transformed to log10, and correlated analysis of variance was performed followed by the test for comparison of average SNK significance ($p < 0.05$) using SIGMASTAT statistical package (1994).

RESULTS AND DISCUSSION

The levels of coli forms at 35 and 45°C, *Salmonella* and *B. cereus* in rice derived samples are shown in Table 1. All this parameters are below the limits established by law RDC No. 12, of January 2001 (Brazil, 2001). Many pathogens can be associated with seeds and grains of rice, harming the health quality of their products (Guimarães et al., 2010). Thus, monitoring the quality of their products is of great importance, specifically in relation to microbiological standards, since these features allow an assessment of the conditions of processing, storage, distribution, service life and the risk to consumer health.

The counting of yeast and filamentous moulds was performed by means of enumeration of fungal propagules and expressed as colony forming units per gram of analyzed sample (CFU/g) (Table 1). The fungal counts varied from 2.96 to 4.17 CFU/g. There was no significant difference between the different marks of corn flour analyzed ($p<0.05$).

The presence of fungi in food can cause modifications in the organoleptic characteristics such as: taste, smell and appearance, leading to a significant decrease in food quality (Cast, 2003). Table 2 show the occurrence of filamentous fungi in rice products sold in commercial establishments from Teresina, PI, Brazil. All analyzed brands, both rice flakes as rice dough, were contaminated by different genera of fungi, some potentially able to produce mycotoxins, which may have a potential risk to human health. 132 fungal colonies were isolated, which were distributed in eight genera of fungi. The genus most frequently isolated was *Aspergillus* spp. and its teleomorphs (35.6%), followed by *Penicillium* spp. (29.5%) and *Cladosporium* spp. (18.2 %). Guimarães et al. (2010), using two techniques for fungal detection,

Table 3. Relative frequency (%) of *Aspergillus* species isolated from rice sub-products intended for human consumption.

***Aspergillus* species**	**No. of strains**	**Frequency (%)**
A. flavus	15	31.9
Eurotium spp.	07	14.9
A.ostianus	07	14.9
A.clavatus	05	10.6
A.fumigatus	03	6.4
A.niger and agregados	03	6.4
A. terreus	02	4.3
A.niveus	02	4.3
A. candidus	01	2.1
A. paradoxy	01	2.1
A.oryzae	01	2.1
Total	47	100

Table 4. Relative frequency (%) of *Penicillium* species isolated from rice sub-products intended for human consumption.

***Penicillium* species**	**No. of strains**	**Frequency (%)**
P. citrinum	21	53.8
P. restrictum	07	17.9
P. corylophilum	03	7.7
P. decumbens	03	7.7
P. implicatum	02	5.1
P. citreonigrum	01	2.6
P. paxilli	01	2.6
P. purpurogem	01	2.6
Total	39	100

found that genera *Penicillium, Aspergillus*, *Cladosporium*, *Fusarium* and *Trichoderma* were present in samples of polished white rice.

Out of 47 *Aspergillus* spp. strains isolated, 7 belong to *Aspergillus* teleomorph Eurotium. The most frequent species was *A. flavus* with 31.9% followed by *A. ostianus* (14.9%). The relative frequencies of species *A. niger* and aggregated (6.4%), *A. fumigatus* (6.4%) were low, however, the presence of such species is significant because these species can produce mycotoxins (Abarca et al., 2001). Other *Aspergillus* species were identified at a lower frequency as *Aspergillus terreus, Aspergillus clavatus, A. terreus, Aspergillus niveus, Aspergillus candidus, Aspergillus paradoxy* and *Aspergillus oryzae*.

Among the *Aspergillus* species, we can observe in Table 3 that the 30% of the strains was *A. flavus*. The presence of *A. flavus* in foods poses a potential hazard because it can cause disease in workers who are directly in contact with it, such as aspergillosis (Akan et al., 2002), allergies and respiratory problems by contact and inhalation of conidia. Also, the potential to produce aflatoxin, if the stored conditions are not appropriate, is significant. All strains of *A. flavus* were analyzed for their ability to produce aflatoxins using two techniques, culture and chromatographic method, but none of the strains evaluated show ability to produce aflatoxins. In a study done by Guimarães et al. (2010), in polished rice and parboiled, it was found that 50% of the strains of *A. flavus* were positive in coconut agar technique in the aflatoxin production (but the ability was not checked by other methods.

According to data from the UN Food and Agriculture Organization (FAO, 2006), with appropriate conditions, species of the genus *Aspergillus* spp. can grow groundnut, corn and other grains and produce mycotoxins. Thus, from the results, it may be suspected that these products in their processing steps have not offered sufficient conditions for the fungi to produce aflatoxins.

Eight *Penicillium* species were observed within a total of 39 strains, *Penicillium citrinum* (53. 8%) was the more frequent, followed by *Penicillium restrictum* (17.9%). Other species were also identified in a lesser frequency as *Penicillium corylophilum, Penicillium decumbens, Penicillium citreonigrum, Penicillium implicatum* and *Penicillium paxilli* (Table 4).

P. citrinum is one of the most common fungal species in Brazilian foods, and the responsible for the citrinin contamination, a toxin nephrotoxic (Oliveira et al., 2006). All strains of *P. citrinum* isolated were tested in their ability to produce citrinin, using the agar coconut technique, none of the strains produced the toxin.

All the samples of rice products were analyzed for the presence of mycotoxins (aflatoxins, ochratoxin A and zearalenone), however, with the method used, the presence of any of them was not evidenced. In a survey carried out by Nunes et al. (2003) in different types of rice (coarse, parboiled and white polished), the authors detected samples contaminated with ochratoxin A and zearalenone. Silva et al. (2008) analyzed the aflatoxin contamination in the rice used in a government department by thin layer chromatography and toxin was not detected, but when high performance of liquid chromatography was used in the same samples, aflatoxins were detected in 23.07% of the samples. In this study, aflatoxins were derived in a post-column electrochemical reactor KOBRACELL mark and screened by fluorescence detection with a wavelength of 425 to 360 nm and B1 and B2, G1 and G2 to 455 nm, and the quantization limit of the technique 0, for each aflatoxin 5 mg/kg.

It is recommended that survey will be conducted routinely in rice, not only fungi but also bacteria, because only with this control, the consumer providing quality products can be guaranteed.

Conclusion

The rice products (rice dough and rice flakes) commercialized in Terezina had satisfactory, hygienically and sanitary conditions by the techniques, the presence of products in the established standard by the relevant legislation was not found. Mycotoxins were not detected in the samples.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Abarca ML, Bragulat MR, Castella g, Cabañes FJ (2001). Current importance of ochratoxin A producing Aspergillus spp. J. Food Prot. 64:903-906.

Akan M, Haziroglu R, Ilhan Z, Sareyyupoglu B, Tunca RA (2002). Case of aspergillosis in a broiler breeder flock. Avian Diseases, 42(2):497-501.

American Public Health Association (APHA) (2005) . Compendium of methods for the microbiological examination of foods. 5 ed. Washington. 676 p.

Boeing CR (2014). Micotoxinas: causa de envenenamento alimentar. Disponível em: <http://www.crq.org.br/solucao/numero18/notícia1.htm>. Acesso em: 28 jul.

Brasil (2014). Agência Nacional de Vigilância Sanitária. Resolução RDC nº 12, de 01 de janeiro de 2001. Aprova o regulamento técnico sobre padrões microbiológicos para alimentos. 2001. Disponível em: <http://e-legis.anvisa.gov.br/leisref/public/showAct.php?id=144>. Acesso em: 26 jul.

Cast (2003). Council for Agricultural Science and Technology. Mycotoxins: risks in plant, animal and human systems. Task Force Report Nº139, Ames, Iowa, USA.

Conab (2014). Companhia Nacional de Abastecimento. Estudos de Prospecção do Mercado Safra 2012/2013. Brasilia, 2012. Disponível em: http://www.conab.gov.br/OlalaCMS/uploads/arquivos/12_09_11_16_41_03_prospeccao_12_13.pdf> . Acesso: 02 out.

Dilkin P, Mallmann CA (2014). Sinais clínicos e lesões causadas por micotoxinas. In: ENCONTRO NACIONAL DE MICOTOXINAS, 11, 2004. Anais. Piracicaba – SP: Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo. Disponível em: <http//www.lamic.ufsm.br>. Acesso em: 20 agost.

Food and Agriculture Organization (FAO) (2014) Rice around the world. 2006. Disponivel em: <http://www.fao.org/rice2004>. Acesso em: 02 agost..

Freitas JR, Schoenau JJ, Boyethko SM, Cyrenne SA (2003) Soil microbial populations, community composition, and activity as affected by repeated applications of hog and cattle manure in eastern Saskatchewan. Can. J. Microbiol. 49, 538-548.

Galvano F (2005). Mycotoxins in the human food Chain. In: Diaz DE The micotoxin blue book. Nottingham:Nottingham University. p. 187-223.

Ghelardi E, Celandroni F, Salvetti S,Barsotti C, Baggiani A, Senesi S (2002). Identification and characterization of toxigenic *Bacillus cereus* isolates responsible for two food-poisoning outbreaks. FEMS Microbiology Letters, 208(1):129-34.

Guimarães ICO, Souza ARM, Cornelio VMO, Pereira J, Villela VA (2010). Identificação de Aspergillus spp. toxigênico em arroz. Ciênc. Tecnol. Aliment., Campinas, 30(Supl.1): 60-62.

Guimarães ICO, Pereira J, Cornelio VMO, Batista LR, Evangelista RM, Ferreira EB (2010). Comparação de metodologias para detecção de fungos em arroz irradiado. Rev Inst Adolfo Lutz.; 69(2):194-200.

Jones FT, Richardson KE (2004). Salmonella in commercially manufactured feeds. Poultry Science. 83:384-91.

Klich MA, Pitt JI (1994). A laboratory guide to common *Aspergillus* species and their teleomorphs. Clayton South: Commonwealth Scientific and Industrial Research.

Lin MT, Dianese JC (1976). A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. Phytopathology, v. 66, n. 12, p. 1466-1469.

Moss MO (1991). Mycology of cereal grain and cereal products. In: Chelkowski J. (Ed.). Cereal grain. Mycotoxins, fungi and quality in drying and storage. Ámsterdam: Elsevier.

Nunes IL, Magagnin G, Bertolin TE, Furlong EB (2003). Arroz comercializado na região Sul do Brasil: Aspectos Micotoxicológicos e Microscópicos. Ciênc. Tecno. Aliment. Campinas, 23(2): 190-194.

Oliveira GR, Ribeiro JMM, Fraga ME, Cavaglieri LR, Direito GM, Keller KM, Dalcero AM, Rosa CAR (2006). Mycobiota in poultry feeds and natural occurrence of aflatoxins, fumonisins and zearalenone in the Rio de Janeiro State, Brazil. Mycopahologia.162:355-362.

Pitt JI (1988). Laboratory guide to commom *Penicillium* species. 2.ed. North Ride: CSIRO Division of Food Processing, 186p.

Pitt JI, Hocking AD (2007). Fungi an food spoilage. London: Blackie Academic & Professional. 536 p. 3ª Ed.

Rodriguez-Amaya DB, Sabino M (2002). Mycotoxins research in Brazil: the last decade in review. Brazilian J. Microbiol. (33(1):1-11.

Santos AB, Stone LF, Vieira NR (1994). A cultura do arroz no Brasil. 2ed. Santo Antonio de Goiás: EMBRAPA Arroz e Feijão; 2006 Sigma Stat for windows version 1.0. Jandel Corporation.

Silva JO, Cândido LM, Novello D, Machado C (2008). Ocorrência de aflatoxinas em arroz consumido por militares do exército brasileiro por cromatografia em camada delgada e cromatografia líquida de alta eficiência. Ciênc. agrotec., Lavras, 32(4):1238-1244.

Soares VLM, Rodriguez-Amaya DB (1989). Survey of aflatoxins, ochratoxin A, zearalenone, and sterigmatocystin in some Brazilian foods by using multi-toxin thin-layer chromatographic method. Journal of Association of Official Analytical Chemists, 72:22-26.

Tanaka K, Sago Y, Zheng Y, Nakagawa H, Kushiro M (2007) Mycotoxins in rice. Int. J. Food Microbiol. 119:59-66.

Yoshisawa T (2001). Mycotoxins analyses for federative republic of Brazil. Japão: Trainig Course, 283 p.

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Full Length Research Paper

Prevalence of some food poisoning bacteria in local and imported retail pork by-products in Egyptian markets

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A very limited research work concerning foods of porcine origin in Egypt were obtained in spite of presence of a considerable swine population and consumers. This study was conducted to investigate the prevalence of food poisoning bacteria isolated from local and imported retail pork by-products in Egyptian markets. A total of 80 pork samples, including 60 local pork by-products and 20 imported ones were used. The isolated bacteria species after biochemical and serological typing were *Escherichia coli* (59) and distributed as *E. coli* O157(27), *E. coli* O146(18) and *E. coli* O111 (14) by 33.75, 22.5 and 17.5%, respectively followed by *Staphylococcus aureus* which was isolated from 23 (28.75%), *Salmonella* spp. was represented by *Salmonella typhimurium* (9) *Salmonella enteritidis* (7) and *Salmonella agona* (4), as 11.25,8.75, and 5%, respectively. Finally, *Listeria monocytogenes* was isolated from 9 samples as 11.25%. The bacterial isolates were sensitive to ciprofloxacin and more resistant to penicillin, gentamicin, amoxicillin and ceftazidime. The bacterial isolation is considerably more in the local pork by-products than the imported samples. On the whole, both types are commonly in permissible limits of the Egyptian food quality standard as the high A.P.C. were *Staphylococci* and *E. coli* followed by *Salmonella* spp., then *L. monocytogenes*. To the best of our knowledge, this is the first report on isolation and identification of food born bacteria from pork by-products in Egypt.

Key words: Pork by-products, local, imported, food poisoning bacteria, Egypt.

INTRODUCTION

Food-borne diseases are an important cause of morbidity and mortality worldwide. Food contamination with anti-biotic-resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other pathogenic bacteria, potentially compromising the treatment of severe bacterial infections (Swartz, 2002). The prevalence of antimicrobial resis-tance among food-borne pathogens has increased during

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recent decades (Threlfall et al., 2000). Commonly, the developing countries have bad raw food hygiene, lack incidence of foodborne disease and antimicrobial resistance epidemiology, thus, management of biological hazards transmitted to humans by food consumption is of major health significance (Thi Thu et al., 2007). Good manufacturing/production practices and various interventions by slaughter and meat processing facilities play a large role in enhancing the safety of meat products. Baseline studies to determine microbial levels of pathogen prevalence can be used to assess the effectiveness of these programs and interventions (Bohaychuk et al., 2011).

Foods of porcine origin are an important vehicles associated with illnesses caused by foodborne pathogens, which lead to the development of antibiotic-resistant pathogens such as *Salmonella* spp., *Escherichia coli*, *Yersinia* spp., Staphylococci, *Listeria monocytogenes* (Wang et al., 2013).

Salmonella species are considered to be among the most important foodborne pathogens in the world and salmonellosis is still one of the most widespread foodborne bacterial illnesses in humans, with clinical manifestations ranging from asymptomatic state to severe disease (Galanis et al., 2006). The majority of infections are associated with the ingestion of contaminated foods such as pork. *Salmonella* in pork carcasses is a result of faecal contamination during slaughtering and processing. In this case, the carrier swine are the main initial source of contamination (Sylvia et al., 2005).

Foods of porcine origin are considered one of the sources of *E. coli* illnesses in humans. Most outbreaks of *E. coli* have been linked with the consumption of undercooked pork by-products; pork sausages and salami (Dias et al., 2013). The ability of *E. coli* to adapt to acid environments has caused this microorganism to be regarded as one of the most dangerous pathogens in fermented pork products. Several studies have shown that *E. coli* is able to survive the processes of fermentation, drying and storage when this microorganism is present in high numbers (Trotz-Williams et al., 2012).

L. monocytogenes poses a serious threat to public health, and the majority of cases of human listeriosis are associated with contaminated food. Pork meat and processed pork products were the sources of outbreaks of listeriosis during the last decade (Thévenot et al., 2006).

In Egypt, a very limited research work concerning epidemiological studies has implicated foods of porcine origin as an important vehicle associated with illnesses caused by foodborne pathogens, which lead to the development of public health hazards.

The present study was undertaken to provide a baseline data for strains isolated from local and imported pork and pork by-products in Egyptian markets.

METHODOLOGY

This study was conducted on 80 pork samples, including 6 types of local pork and pork by-products (n = 60) and 5 types of imported pork by-products (n = 20), purchased from pork retail markets. Samples were double-bagged at the source, refrigerated until delivery to the laboratory and then handled in such a manner as to prevent cross-contamination, and were examined within 1 day of purchase; they were chopped into small pieces, and 25 g from each sample was transferred to 225 ml of 1% buffered peptone-water and incubated for 24 h at 25 or 37°C.

Cultures were diluted to 10^{-4} in 0.1% peptone-water, and 100 µl volumes of different dilutions were spread on different specific agar media. The plates were then incubated at 37°C for 48 h, after which colonies were enumerated and the total bacterial (colony forming units) were calculated, as described by Azza et al. (2014).

Total plate count at 35°C (mesophiles)

The pouring technique recommended by AOAC (2000) was applied. 1 ml of each dilution was separately pipetted in sterile Petri-dishes. 15 ml of melted standard plate count agar (SPCA;Oxiod;CM325) at 42-45°C were poured; thoroughly mixed and then left to solidify. The inoculated plates were incubated at 35°C for 48 ± 2 h. The average number of colonies was determined and the aerobic plate count per gram was calculated as follows:

Mesophilic plate count/g/org. = No. of colonies × dilution.

Total plate count at 25°C (psychrotrophic bacteria)

The same technique of the pouring method was done as previously mentioned in mesophiles but the inoculated plates were incubated at 25°C for 48 h. The number of colonies/g was calculated in countable plates as follows:

Psychrotrophic count/g/org. = No. of colonies × dilution

Isolation and identification

The remaining TSB in the containers was incubated at 37°C for 12 h. Thereafter, the broth cultures were plated on selective and/or differential media, namely blood agar, MacConkey agar, Eosin methlene blue (EMB), xylose lysine desoxycholate agar, *Salmonella Shigella* agar (S.S. agar) mannitol salt agar and PALCAM agar. The plates were incubated at 37°C 24 h. Bacterial colonies in each medium were then characterized on the basis of colonial, cellular morphology and staining characteristics. On this basis, the colonies were categorized as Gram positive, catalase positive cocci; Gram positive short rods and Gram negative bacilli according to Koneman et al. (1983).

Biochemical identification

Organisms in each category were then identified, when possible, on the basis of biochemical characteristics by applying catalase activity test, IMVC reactions tests, hydrogen sulfide production (triple sugar iron agar, TSI), hydrolysis of urea, sugar fermentation, nitrate reduction and detection of motility according to Carter and Cole (1990).

Serological identification

The somatic (O) antigen of *E. coli* was determined by slide agglutination test according to Edwards and Ewing (1972), while

Flagellar (H) antigen sereotyping was carried out according to Davies and Wray (1997). Anti-O-sera were obtained from DENKA SEIKEN CO LTD Tokyo, Japan. *Salmonella* spp. was serotyped according to Bale et al. (2007). *Listeria* spp. was serologically identified with factor sera according to Schnberg et al. (1989).

Sensitivity test for antibiotics

It was carried out according to the National Committee for Clinical Laboratory Standards, 2000.

Preparation of standard suspension

Some of typical colonies of each isolate were suspended in Mueller-Hinton broth and incubated at 37°C for 8 h till its turbidity exceeds the turbidity of standard McFarland tube No. 0.5.

Inoculation of the test plates

A sterile cotton swab was dipped into standardized bacterial suspension. The swab was then used to streak the dried surface of Mueller-Hinton agar plate in three different planes by rotating the plates to be sure for even distribution of the inoculums.

Placement of the discs

The antimicrobial discs were placed on the inoculated place using gentle pressure by sterile pointed forceps on the agar to ensure complete contact with the surface. Then the plates were incubated at 37°C for 24 h.

Reading of the results

The degree of sensitivity was estimated by measuring the visible clear zone of inhibition produced by the diffusion of the used antimicrobial disc into the surrounding medium. Interpretation of the results was done according to the National Committee for Clinical Laboratory Standards (2000).

RESULTS AND DISCUSSION

From the results presented in the Tables 1 and 2, the bacterial isolation is considerably more in the local pork and pork by-products than the imported samples. On the whole, both types are commonly in permissible limits of the Egyptian food quality standard as the high T.P.C. were *Staphylococci* and *E. coli* followed by *Salmonella* spp. then *L. monocytogenes*. Manguiat and Fang (2013) reported high levels of aerobic plate count, *E. coli* and *S. aureus*. The highest counts obtained were 8.2, 5.4, 4.4 log and 3.9 log cfu g^{-1}, respectively, *Salmonella* was found in 8% of the samples.

Table 3 shows the bacteria species isolates after biochemical and, serological typing were *E. coli* (59), and distributed as *E. coli* O157(27), *E. coli* O146 (18) and *E. coli* O111(14) by 33.75,22.5 and 17.5%, respectively.

Shiga toxin-producing *E. coli* (STEC) strains are food-borne pathogens that are an important public health concern. STEC infection is associated with severe clinical diseases in human beings, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which can lead to kidney failure and death. Tseng et al. (2014) stated that a number of STEC outbreaks and HUS cases have been attributed to pork products in spite of the role that swine play in STEC transmission to people and the contribution to human disease frequency requires further evaluation.

Magwedere et al. (2013) investigated STEC O-groups, responsible for the majority of *E. coli* infections in human beings, in retail pork meat (n = 16), and represented 8 samples (9%). Johnson et al. (2005) concluded that ground pork may be an important vehicle for community-wide dissemination of *E. coli* and Rode et al. (2012) mentioned that dry-fermented sausages are considered possible risk pork products regarding STEC.

S. aureus was isolated from 23 as 28.75%, and this result was nearly similar to the results obtained by Koláčková et al. (2014) who evaluate the contamination of raw pork meat with *S. aureus* in the retail market and found that 21.8% were found to be positive, and Atanassova et al. (2001) who detected *S. aureus* as 25.9%. We observed that the isolation of *S. aureus* from salami and mortadella was the least and this may be attributed to their low pH and proved to be a difficult environment for the survival of *S. aureus* (Wallin-Carlquist et al., 2010).

The isolated *Salmonella* spp. were represented by Salmonella *typhimurium* (9) *Salmonella entertidis* (7) *and Salmonella agona* (4), as 11.25, 8.75 and 5%, respectively. These results agreed with that of Kerouanton et al. (2013 who mentioned *S. typhimurium* as the most often isolated serotypes in pigs, pork and pork by-products, also Lin et al. (2014) isolated *S. enteritidis* from pork.

Our study shows that *L. monocytogenes* was isolated from nine samples as 11.25%, but in very low colony count and this agreed with the result obtained by Ristori et al. (2014) who mentioned that the *L. monocytogenes* populations were $<10^2$ cfu/g in the majority of samples.

Finally, the obtained results of the study revealed that the porcine liver and kidney are the highest bacterial colony populations among the samples followed by minced pork and these results are supported by those obtained by Sasaki et al. (2013) who suggested that the consuming swine livers and kidneys without proper heat treatment may increase the risk of foodborne illnesses.

As shown in Table 4, 100 and 30% of the *S. aureus* isolates were resistance to penicillin and amoxicillin respectively, while 91 and 83% were sensitive to ciprofloxacin and ceftazidime respectively. These results agree with Espinosa et al. (2011) who mentioned that the rate of ciprofloxacin and amoxicillin sensitivity for *S. aureus* is 100 and 60%, respectively. The isolates showed 100% resistance to penicillin.

As shown in Tables 5, 6 and 7, the *E. coli* isolates were more resistant to amoxicillin and ceftazidime, and more

Table 1. Enumeration of the isolated bacteria from retail local pork and pork by-products.

Sample	Microorganism							
	Staphylococci		*Escherichia coli*		*Salmonella spp.*		*Listeria* spp.	
	T.P.C.	No	T.P.C	No	T.P.C	No	T.P.C	No
Salami	$4x10^2$	2	$1x10^2$	8	2 x10	1	0	0
Mortadella	$4x10^2$	2	$1.2x10^2$	2	2 x10	1	0	0
Sausage	$3x10^3$	1	$3 x10^2$	1	$1x10^2$	2	10	2
Minced meat	$4 x10^3$	3	$3 x10^3$	0	$1x10^3$	2	14	2
Liver	$8 x10^3$	5	$3 x10^3$	3	$2x10^2$	2	18	2
Kidney	$6 x10^4$	3	$3 x10^3$	3	$3 x10^2$	3	22	3

Table 2. Enumeration of the isolated bacteria from retail imported pork by-products.

Sample	Microorganism							
	Staphylococci		*Escherichia coli*		*Salmonella spp.*		*Listeria* spp.	
	T.P.C.	No	T.P.C.	No	T.P.C.	No	T.P.C.	No
Salami	$1x10^2$	1	$1x10^2$	2	1x10	1	0	0
Mortadella	4 x10	1	1 x10	1	1 x10	1	0	0
Bavarian sausage	$3 x10^4$	3	$3 x10^2$	4	$3 x10^2$	4	8	1
Canadian bacon	$2 x10^2$	1	$2 x10^2$	3	2 x10	1	0	0
Smoked bacon	$3 x10^3$	1	$2 x10^2$	2	1 x10	1	0	0

Table 3. Identification of the isolated bacteria from retail local, imported pork by-products.

Microorganism	(n=80)
Staph.aureus	18.75%
E.coli O157	33.75%
E.coli O146	22.5%
E.coli O111	17.5%
Salmonella typhimurium	11.25%
Salmonella enteritidis	8.75%
Salmonella agona	5%
Listeria monocytogenes	11.25%

Table 4. Antibiogram sensitivity test of *S. aureus* isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	7	30%	3	13%	13	57%
Penicillin	23	100%	0	0%	0	0%
Ciprofloxacin	0	0%	2	8.5%	21	91%
Ceftazidime	2	8.5%	2	8.5%	19	83%

The percent was calculated according to the total number of *S. aureus* isolates (n=23).

sensetive to gentamycine and ciprofloxacin.These results greatly agree with that of Espinosa et al. (2011) who mentioned that *E. coli* isolates are resistant to amoxicillin (70%) and were sensitive to ciprofloxacin (100%).

As shown in Table 8, 9 and10, the *Salmonella* isolates were more resistant to amoxicillin, gentamycine and

Table 5. Antibiogram sensitivity test of *E. coli* O157 isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	20	74%	4	15%	3	11%
Ciprofloxacin	14	52%	3	11%	10	37%
Gentamicin	9	34%	2	7%	16	59%
Ceftazidime	19	70%	3	11%	5	19%

The percent was calculated according to the total number of *Escherichia coli* O157 isolates (n=27).

Table 6. Antibiogram sensitivity test of *E. coli* O146 isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	11	61%	3	17%	4	22%
Ciprofloxacin	8	45%	4	22%	6	33%
Gentamicin	2	11%	2	11%	14	78%
Ceftazidime	10	55.5%	1	5.5%	7	39%

The percent was calculated according to the total number of *Escherichia coli* O146 isolates (n=18).

Table 7. Antibiogram sensitivity test of *Escherichia coli* O111 isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	9	65%	3	21%	2	14%
Ciprofloxacin	6	43%	3	21%	5	36%
Gentamicin	0	0%	3	21%	11	79%
Ceftazidime	8	58%	4	28%	2	14%

The percent was calculated according to the total number of *Escherichia coli* O111 isolates (n=14).

Table 8. Antibiogram sensitivity test of *Salmonella typhimurium* isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	9	0%	0	0%	0	0%
Ciprofloxacin	2	22%	0	0%	7	78%
Gentamicin	8	89%	1	21%	0	0%
Ceftazidime	3	33%	1	11%	5	56%

The percent was calculated according to the total number of *Salmonella typhimurium* isolates (n=9).

Table 9. Antibiogram sensitivity test of *Salmonella enteritidis* isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	6	86%	1	14%	0	0%
Ciprofloxacin	3	43%	1	14%	3	43%
Gentamicin	5	72%	1	14%	1	14%
Ceftazidime	4	58%	2	28%	1	14%

The percent was calculated according to the total number of *Salmonella entertidis* isolates (n=7).

Table 10. Antibiogram sensitivity test of *Salmonella agona* isolates.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	4	100%	0	0%	0	0%
Ciprofloxacin	0	0%	0	0%	4	100%
Gentamicin	3	79%	1	21%	0	0%
Ceftazidime	4	100%	0	0%	0	0%

The percent was calculated according to the total number of *Salmonella agona* isolates (n=4).

Table 11. Antibiogram sensitivity test of *Listeria monocytogenes*.

Antibacterial agent	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Amoxicillin	1	11%	1	11%	7	78%
Penicillin	0	100%	1	11%	8	89%
Ciprofloxacin	5	55.5%	3	33.5%	1	11%
Ceftazidime	6	67%	1	11%	2	22%

The percent was calculated according to the total number of *Listeria monocytogenes* isolates (n=9).

ceftazidime, and more sensetive to ciprofloxacin. These results nearly agree with that of Espinosa et al. (2011) who mentioned that *Salmonella* isolates were100% resistant to amoxicillin and 100% sensitive to ciprofloxacin. Also, Dechen et al. (2011) and Sang et al. (2012) mentioned that *Salmonella* isolates were 100% sensitive to ciprofloxacin.

As shown in Table 11, the *L. monocytogenes* isolates were more resistant to ciprofloxacin and ceftazidime, and more sensetive to penicillin and amoxicillin and these findings agree with those of Moreno et al. (2014) who mentioned that isolates of *L. monocytogenes* were susceptible to penicillin and possessed at least intermediate resistance to fluoroquinolones.

Conclusion and recommendation

Data regarding the bacteriological count and isolation in APC, *S. aureus*, *E. coli*, *Salmonella* spp. and *L. monocytogenes* from local and imported pork and pork by-products were relatively lower than the Egyptian food quality standard. Regardless, samples were found to be satisfactory due to low levels of aerobic plate count, but the attention should be given to the antibiotic resistant isolates. Generally,the total bacterial counts were lower in processed, heat treated pork by-products than the raw and porcine organs, so, proper heating and processing of pork and pork by-products is recommended to minimize the public hazards. The obtained data seemed to be firstly described in Egyptian retailed pork by-products and need more investigations and studies.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Atanassova V, Meindl A, Ring C (2001). Prevalence *of Staphylococcus aureus* and staphylococcal enterotoxins in raw pork and uncooked smoked ham--a comparison of classical culturing detection and RFLP-PCR. Int. J. Food Microbiol. 68(1-2):105-113.

Azza SM Abuelnaga, Samy AA, Bakry MA, Hakim AS (2014). Bacteriological assay for the Egyptian currency collected from veterinary field. Int. J. Microbiol. Res. 5 (1):48-53.

Bale JA, de Pinna EM, Threlfall EJ, Ward LR (2007). Kauffmann-White Scheme -: *Salmonella* Identification - Serotypes and Antigenic Formulae. London: Health Protection Agency.

Bohaychuk VM, Gensler GE, Barrios PR (2011). Microbiological baseline study of beef and pork carcasses from provincially inspected abattoirs in Alberta, Canada. Can. Vet. J. 52(10):1095-100.

Carter GR, Cole JR (1990). Diagnostic procedures In Veterinary Bacteriology and Mycology. 5th ed. Academic press. Unc. Harcourt Brance Jovanovich Publishers, London, New York.

Davies RH, Wray C (1997). Immunomagnetic separation for enhanced flagellar antigen phase inversion in *Salmonella*: Bacteriology Department,Central Veterinary Laboratory, Addlestone, Surrey, UK. Lett. Appl. Microbiol. 24:217-220.

Dechen TC, Chanchal L, Pal R, Kar S (2011). Performance standards for Bacteriological profile of septicemia and the risk factors in neonates and infants in Sikkim. J. Glob. Infect. Dis. 3(1):42-45.

Dias FS, Duarte WF, Santos MR, Ramos EM, Schwan RF (2013). Screening of *Lactobacillus* isolated from pork sausages for potential probiotic use and evaluation of the microbiological safety of fermented products. J. Food Prot. 76(6):991-998.

Edwards PR, Ewing WH (1972). Identification of *Enterobacteriacae*. Minneapolis,Burgess Publishing Co., Burgess Publishing CP. Atlanta USA 3 rd ed. pp. 709.

Espinosa CJ, Cortés JA Castillo JS, Leal, AL (2011). Systematic review of antimicrobial resistance among Gram positive cocci in hospitals in Colombia. Biomedica 31(1): 27-34.

Galanis E, Lo Fo Wong DM, Patrick ME, Binsztein N, Angulo FJ, WegenerHC (2006). Web-based surveillance and global *Salmonella* distribution, 2000-2002. Emerg. Infect. Dis. 12: 381-388.

Johnson JR, Kuskowski MA, Smith K, O'Bryan TT, Tatini S (2005). Antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in retail foods. J. Infect. Dis. 191(7):1040-1049.

Kerouanton A, Rose V, Weill FX, Granier SA, Denis M (2013). Genetic diversity and antimicrobial resistance profiles of *Salmonella enterica* serotype derby isolated from pigs,pork, and humans in France. Foodborne Pathog. Dis. 10(11):977-984.

Koláčková I, Koukalová K, Karpíšková R (2014). Occurrence and characteristic of *Staphylococcus aureus* in pork meat. Epidemiol Mikrobiol. Imunol. 63(3):191-194.

Koneman EW, Allen SD, Dowell VR, Sommers HM (1983). Color Atlas, Text book and Diagnostic Microbiology. 2nd ed. JB Lippincott Company, New York.

Lin D, Yan M, Lin S, Chen S (2014). Increasing prevalence of hydrogen sulfide negative *Salmonella* in retail meats. Food Microbiol. 43:1-4.

Magwedere K, Dang HA, Mills EW, Cutter CN, Roberts EL, DeBroy C (2013). Incidence of Shiga toxin-producing *Escherichia coli* strains in beef, pork, chicken, deer, boar, bison, and rabbit retail meat. J. Vet. Diagn. Invest. 25(2):254-258.

Manguiat LS, Fang TJ (2013). Microbiological quality of chicken- and pork-based street-vended foods from Taichung, Taiwan, and Laguna, Philippines. Food Microbiol. 36(1):57-62.

Moreno LZ, Paix?o R, Gobbi DD, Raimundo DC, Ferreira TP, Moreno AM, Hofer E, Reis CM, Matté GR, Matté MH (2014). Characterization of antibiotic resistance in Listeria spp. isolated from slaughterhouse environments, pork and human infections. J. Infect. Dev. Ctries. 8(4):416-423.

National Committee for Clinical Laboratory Standards (2000). Performance standards for Bacteriological profile of antimicrobial susceptibility testing. Informational supplement Villanova, PA.

Official Methods of Analysis (AOAC) (2000). 17th ed. Gaithersburg, Maryland, USA, AOAC International.

Ristori CA, Rowlands RE, Martins CG, Barbosa ML, Yoshida JT, de Melo Franco BD (2014). Prevalence and Populations of *Listeria monocytogenes* in Meat Products Retailed in Sao Paulo, Brazil. Foodborne Pathog. Dis. 11(12):969-973.

Rode TM, Holck A, Axelsson L, Hý M, Heir E (2012). Shiga toxigenic *Escherichia coli* show strain dependent reductions under dry-fermented sausage production and post-processing conditions. Int. J. Food Microbiol. 155(3):227-233.

Sang WK, Oundo V, Schnabel, D (2012). Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhea in four provinces of Kenya. J. Infect. Dev. Ctries. 23(7):572-528.

Sasaki Y, Haruna M, Murakami M, Hayashida M, Ito K, Noda M, Yamada Y (2013). Prevalence of *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, and *hepatitis* E virus in swine livers collected at an abattoir. Jpn. J. Infect. Dis. 66(2):161-164.

Schnberg A1, Teufel P, Weise E (1989). Serovars of *Listeria monocytogenes* and *Listeria innocua* from food. Acta Microbiol. Hung. 36(2-3):249-253.

Swartz MN (2002). Human diseases caused by foodborne pathogens of animal origin. Clin. Infect. Dis. 34:111-122.

Sylvia V, Ana V, Herrera-Len S, Javier P, Carvajal A, Aurora EM (2005). *Salmonella Derby* clonal spread from pork. Emerg. Infect. Dis. 11(5): 694-698.

Thévenot D, Dernburg A,Vernozy-Rozand C (2006). An updated review of *Listeria monocytogenes* in the pork meat industry and its products. J. Appl. Microbiol. 101(1):7-17.

Thi Thu HV, George M, Taghrid I, Linh TT, Peter JC (2007). Detection of *Salmonella* spp. in retail raw food samples from Vietnam and characterization of their antibiotic resistance. Appl. Environ. Microbiol. 73(21):6885-6890.

Threlfall EJ, Ward LR, Frost JA, and Willshaw GA (2000). The emergence and spread of antibiotic resistance in food-borne bacteria. Int. J. Food Microbiol. 62:1-5.

Trotz-Williams, LA, Mercer NJ, Walters JM, Maki AM, Johnson RP (2012). Pork implicated in a Shiga toxin producing *Escherichia coli* O157:H7 outbreak in Ontario, Canada. Can. J. Public Health 103(5):322-326.

Tseng M, Fratamico PM, Manning SD, Funk JA (2014). Shiga toxin-producing *Escherichia coli* in swine: the public health perspective. Anim. Health Res. Rev. 8:1-13.

Wallin-Carlquist N, Márta D, Borch E, Rådström P (2010). Prolonged expression and production of *Staphylococcus aureus* enterotoxin A in processed pork meat. Int. J. Food Microbiol. 31:141 Suppl. 1:S69-74.

Wang JP, Yeh KS, Hsieh MW, Fang CY, Chen, ZW, Lin JH (2013). Pathogenic microbiological baseline survey of pork carcasses in Taiwan. J. Food Prot. 76(6):1046-1050.

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Full Length Research Paper

Bioactivity of *Zingiber officinale* and *Piper nigrum* plant extracts in controlling post-harvest white yam (*Dioscorea rotundata*) tuber rot fungi

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Rotten white yam (*Dioscorea rotundata*) tuber samples were collected from farms in three communities. Nine fungal organisms were isolated by direct tissue plating on potato dextrose agar medium. These were: *Fusarium oxysporum, Aspergillus flavus, Aspergillus niger, Penicillium* spp*., Lasiodiplodia theobromae, Trichoderma viride, Rhizopus* spp*., Pestalotia guepini* and *Alternaria solani*. Each of these isolates caused rot lesions when inoculated into healthy yam tubers. These organisms were re-isolated, identified and compared with the initial isolates to confirm their pathogenicity. *L. theobromae, A. niger, Rhizopus* sp. and *A. solani* were the most frequently isolated fungi species from the rotten yam tuber samples, with frequency of isolation of 30.07, 16.08, 16.08 and 12.59%, respectively. *Zingiber officinale* rhizome and *Piper nigrum* seeds were tested against *L. theobromae* and *F. oxysporum in vitro*. The botanical extracts were prepared by cold water extraction method at a concentration of 60% w/v. The two extracts showed significance in inhibiting the growth of the two fungi when data was statistically analysed (p = 0.05) using Genstat 9.2 package. This implies that they have some anti-fungal properties which need to be further investigated *in vivo* to establish their suitability in protecting yam tubers from storage rot. However, *P. nigrum* showed to be much stronger than ginger in terms of bioactivity.

Key words: *Zingiber officinale*, *Piper nigrum*, bioactivity, yam tuber rot, rot fungi.

INTRODUCTION

Rot is a major factor limiting the post-harvest life of yams and losses can be as high as 60% in storage (Adesiyan and Odihirin, 1975). Losses due to post-harvest rot significantly affect farmers' and traders' income, food security and seed yams stored for planting. The quality of yam tubers is affected by rot which makes them unappealing to consumers.

Some white yam varieties like 'pona' that are preferred by most consumers in Ghana, do not store for a long time due to attack by rot organisms. Because of their poor storability, farmers sell produce just after harvest to avoid losses, and this result in low income and reduced profits.

Nine fungal species including *Aspergillus flavus, Aspergillus niger, Lasiodiplodia theobromae, Fusarium culmorum, Fusarium oxysporum, Penicillium brevi-compactum, Penicillium oxalicum* and *Rhizopus*

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stolonifer, have been identified to be associated with yam tuber rot in Ghana (Aboagye- Nuamah et al., 2005). Ezeibekwe and Ibe (2010) reported that *F. oxysporium*, *L. theobromae* and *F. solani* were associated with yam rot diseases in Nigeria.

Fungal pathogens causing rot in yam often gain entry into tubers through wounds caused by insects, nematodes or poor handling before, during and after harvest (Amusa et al., 2003). Rot of fleshy parts of plants that develop as tissues are disintegrated by the action of microorganisms. Extra-cellular enzymes such as hydrolases and lyases are produced in advance of fungal hyphae of the attacking pathogens. The affected tubers become hydrotic and soft, turn brown, emit offensive odour and exhibits a sharp demarcation between a healthy intact tissue and a diseased tissue.

Some of the control measures studied over the years include minimising physical damage of tubers, the use of chemicals, the use of crop rotation, fallowing and planting of healthy materials, destruction of infected crop cultivars, wood ash application and breeding for resistance (Oduro et al., 1991; Nyadanu et al., 2014). Some plants possess fungicidal properties.

Okigbo and Nmeka (2005) showed that extract of *Xylopia aethiopica* and *Zingiber officinale* controlled post-harvest yam rot. Pesticides of plant origin are specifically more biodegradable, readily available, cheaper and environmentally friendly than synthetic chemicals. In this report, the bioactivity of the extracts of *Z. officinale* rhizome and *P. nigrum* seed in controlling the growth of some yam tuber rot fungi were studied.

MATERIALS AND METHODS

Collection of diseased yam tubers

About twenty rotten tubers of white yam ‘pona’ were collected randomly from farms in three districts, namely Kintampo North, Wenchi and Tain districts and sent to the Plant Pathology Laboratory, CSIR-Crops Research Institute, Kumasi, in polythene bags. Collected samples were kept in a refrigerator at 4°C until required.

Isolation of fungal species from rotten yam tubers

Pieces of diseased tissues (50 mm^3 average) cut from the periphery of the disease lesion on the tubers with a sterilized knife were surface-sterilized in 5% sodium hypochlorite solution for 2-3 min. The surface sterilized diseased tissues were washed three times using sterile distilled water. The tissues were allowed to dry in a sterile Lamina flow chamber (BASSAIRE, Duncan Road, Swandick, Southampton, SO3 7ZS) for about 30-45 min. The dried disease tissues were plated on potato dextrose agar (PDA) medium (Merck; Merck KGaA, 64271 Darmstadt, Germany). Two days after incubation, mycelia that grew from the plated yam tissues were sub-cultured onto fresh PDA. Further sub-culturing was carried out until pure cultures of single isolates were obtained. From these pure cultures, inocula of the different fungal species were obtained for the pathogenicity tests. Frequency of occurrence for each organism was determined by calculating the number of colonies of a fungus out of the total number of fungal colonies, expressed as a percentage.

Identification of fungal isolates

Characteristics of fungal isolates from rotten yam tubers such as colour, pigment production, colony texture, spore or conidia-producing structures and spore shapes were documented. The characteristics were observed from fungal mycelia grown on PDA for one week or more, depending on the fungal species. Spore and mycelium characteristics were studied using the compound microscope. These characteristics were used in identifying the fungal organisms to the species level, following standards described by Mathur and Kongsdal (2003) and Barnett and Hunter (1972).

Pathogenicity test

One week old pure cultures of the fungal isolates obtained from rotten yam tubers produced on PDA were the source of inocula for the pathogenicity studies. Middle portion of healthy yam tubers (average 40 cm long) of ‘pona’ were inoculated with the fungal isolates identified (4cm interval). A 5-mm diameter cork borer was used to remove discs (1 cm thick) from the yam tuber surface after surface sterilization of the tubers with 5% sodium hypochlorite solution. The 5-mm diameter cork borer (sterilized by dipping in 100% alcohol followed by flaming) was used to cut plugs from the one week old cultures of the fungal isolates to be tested. These fungal plugs were put in the holes created in the yam tubers after which the removed yam tuber discs were used to plug the holes. Melted candle wax from a burning candle was used to seal the edges of the replaced yam discs. This process prevented contamination by other microbes. Each fungal isolate was replicated three times (on three different yam tubers) in a complete randomised design. Controls were set up whereby no fungal organism was placed in the hole. These activities were carried out inside a sterile hood. After 10 days of inoculation, the inoculated wholes were cut cross-sectionally to observe rot infection by inoculated fungi.

Preparation of plant extracts

Cold water extraction method was used for the preparation of the plant extracts. Fresh rhizomes of *Z. officinale* (ginger) and seeds of *P. nigrum* (black pepper) were washed thoroughly with distilled water. These were further blended into a fine paste separately for each botanical with a blender (Binatone, BLG-401, Hong Kong) at a speed of 4000 rpm for five to ten minutes. Extract concentration of 60% (w/v) was obtained by adding 40 ml of sterile distilled water to 60 g of each botanical paste in a beaker with vigorous stirring.

Anti-fungal bioactivity of plant extracts *in vitro* on yam rot organisms

Two test fungi, *L. theobromae* and *F. oxysporum*, obtained from rotten yam tissues, were used in this experiment. Surface coating of potato dextrose agar (PDA) medium with botanical extracts was the method used to investigate the bioactivity of the extracts. PDA medium was prepared by dissolving 39 g in one litre sterile distilled water and autoclaved at 121°C and 15 psi for 15 min. The medium was poured into sterilized Petri dishes and allowed to solidify. Five hundred microlitres (500 µl) of each botanical extract preparation was spread thinly on the surface of the PDA in Petri dishes. The extract was allowed to dry and the medium inoculated centrally with

(a) *Fusarium oxysporum* (b) *Pestalotia guepini* (c) *Alternaria solani*

Plate 1. Conidia of some fungi identified (from rotten yam tissues) under compound microscope (x40/0.65 magnification).

Plate 2. A cross-section of a tuber of yam inoculated with fungus showing rot lesion.

discs (5 mm diameter) obtained from one-week-old cultures of the test fungi, *B. theobromae* and *F. oxysporum*. Three replications were set for each treatment. Controls were set up in which PDA with no botanical extract were inoculated with test fungi. The organisms were incubated at 26-28°C and measurement of mycelial growth as radius of a growing fungal colony was undertaken and recorded at intervals of twenty-four hours using a ruler. All the analyses were done in triplicates and data obtained were statistically analysed using Genstat (Relaese 9.2) statistical package. Analysis of variance (ANOVA) test and least significant difference at 5% were used to compare the treatment values. Percent growth inhibition of fungal organisms due to the plant extracts was calculated and graphically presented to show the fungicidal action of the plant extracts using the formula of Pandey et al. (1982).

RESULTS

Rot fungi identified

Based on cultural and microscopic characteristics of the cultures, the nine isolates of fungi obtained from rotten tubers were identified as *F. oxysporum, A. flavus, A. niger, Penicillium* sp., *Lasiodiplodia theobromae, T. viride, Rhizopus* sp., *Pestalotia guepini and Alternaria solani*. Plate 1 shows the conidia of some of the fungal isolates from rotten yam tissues produced on PDA.

Each of these isolates was able to cause rot lesions when inoculated into healthy yam tubers (Plate 2). *L. theobromae, A. niger, Rhizopus* spp. and *A. solani* were the most frequently isolated fungal species from the rotten yam tubers collected from the study districts. The frequency of isolation was in the order of 30.07, 16.08, 16.08 and 12.59%, respectively.

Anti-fungal bioactivity of plant extracts against *L. theobromae*

Table 1 shows the mean mycelial growth (mm) of *L. theobromae*. The statistical analysis (ANOVA) showed that there were significant differences among the treatments in the experiment. Mycelial growth of *L. theobromae* on the two plant extract amended PDA were significantly different from the control. However, there was a significant difference between ginger and black pepper at 72-h period although there was no difference between them at 24 and 48 h periods.

After 24 h of incubation, *Z. officinale* rhizome extract inhibited growth of *L. theobromae* by 76.12% when compared with the control. This bioactivity declined to 70.16% at the end of 48 h period and reduced to 64.64%, 72 h after incubation. *P. nigrum* extract at a concentration of 60% (w/v) inhibited growth of *L. theobromae* by 83.58, 80.65 and 81.23% after 24, 48 and 72 h incubation, respectively (Figure 1).

Anti-fungal bioactivity of plant extracts against *F. oxysporum*

Similarly, there was significant difference among the treatments according to the ANOVA results. The two plant extracts showed significant differences from the control. The differences among the plant extracts were realised at 72 and 96 h period (Table 2).

The percent growth inhibition of *F. oxysporum* by *Z.*

Table 1. Effect of plant extracts on mean mycelial growth (mm) of *L. theobromae.*

Treatment	Incubation period (hours)		
	24	48	72
Control	22.33±1.16[a]	41.33±0.58[a]	60.33±2.31[a]
Ginger	5.33±1.53[b]	12.33±2.31[b]	21.33±2.52[b]
Black pepper	3.67±3.22[b]	8.00±4.36[b]	11.67±4.16[c]
LSD(0.05) = 4.966	SED (0.05) = 2.343	CV% = 13.9	

*Values followed by the same letter within a column are not significantly different (p=0.05).

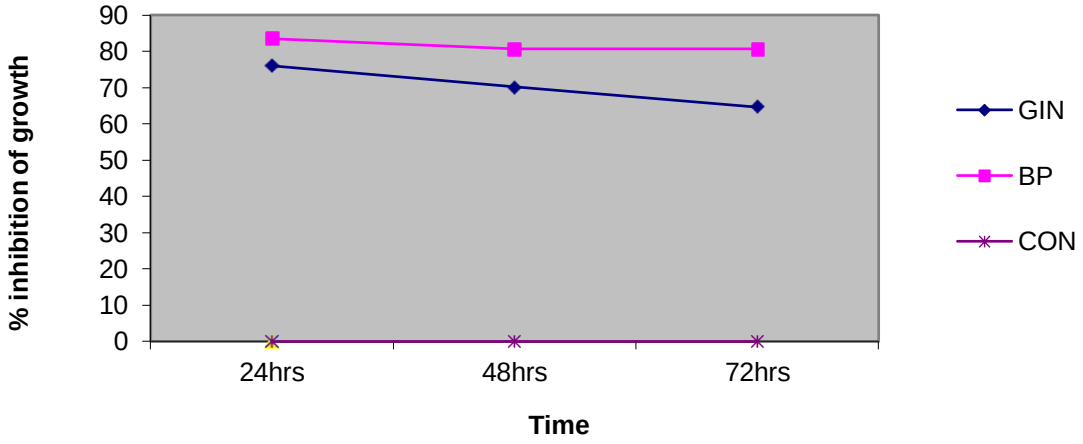

Figure 1. Percent growth inhibition of *B. theobromae* by plant extracts.

Table 2. Effect of plant extracts on mean mycelial growth (mm) of *F. oxysporum.*

Treatment	Incubation period (hours)			
	24	48	72	96
Control	2.00±1.00[a]	10.33±1.53[a]	14.67±2.08[a]	19.33±2.52[a]
Ginger	0.00±0.00[b]	0.00±0.00[b]	1.67±0.58[b]	5.67±1.16[b]
Black pepper	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[c]
LSD (0.05) = 1.571	SED (0.05) = 0.758	CV% = 20.7		

*Values followed by the same letter within a column are not significantly different (p=0.05).

officinale extract (60% concentration) after 24 and 48 h incubation was 100%. Inhibition was 88.64 and 70.69% at the end of 72 and 96 h, respectively. One hundred percent (100%) growth inhibition of *F. oxysporum* was achieved with *P. nigrum,* even at 96 h of incubation (Figure 2).

DISCUSSION

Seven of the identified fungi in this work have been isolated and identified to be rot-causing organisms in other research works done (Okigbo and Ikediugwu, 2002; Aboagye-Nuamah et al., 2005). *L. theobromae* and *Penicillium oxalicum* were reported to cause dry rot of yam (IITA, 1993), whilst *Rhizopus* spp. causes soft rot. These fungi are soil-borne pathogens and this confirms that soils adhering to harvested tubers contain many microorganisms that could be pathogenic to the tubers (Ezeibekwe and Ibe, 2010).

Pesticides of plant origin are known to be more specific, biodegradable, cheaper, more readily available and environmentally friendly than synthetic chemicals. The efficacy of the two botanical extracts (*Z. officinale* and *P. nigrum*) in controlling yam tuber rot fungi was significant. This confirms the work done by Okigbo and

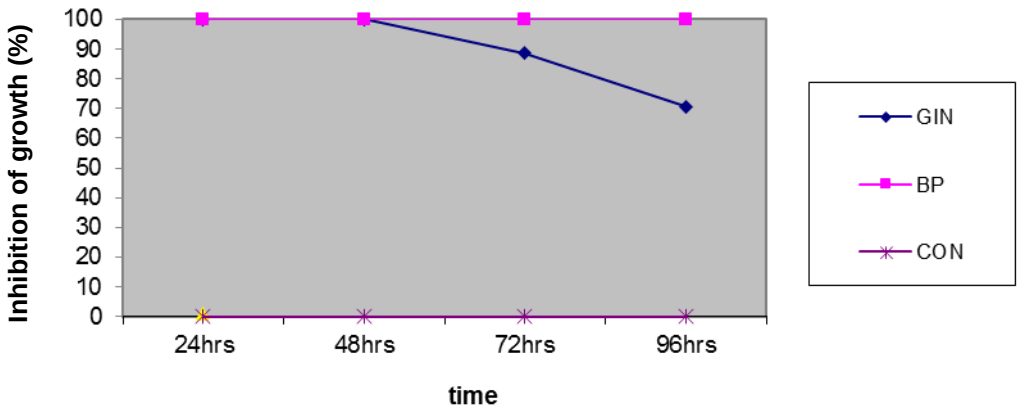

Figure 2. Percent growth inhibition of *F. oxysporum* by plant extracts.

Nmeka (2005) that *Z. officinale* suppresses the growth of rot fungi in culture. *Z. officinale* contains an active ingredient called *gingerol*. Ginger extracts have been shown to possess a broad range of biological activity against fungi (Foster and Yue, 1992). *P. nigrum* has shown to possess anti-fungal properties (Kuhn and Hargreaves, 1987). In this study *P. nigrum* exhibited a stronger and persistent bioactivity as compared to *Z. officinale*.

Conclusion

In conclusion, plant extract based technologies can be developed in the near future to control these organisms on yam tubers. It is recommended that the anti-fungal properties of the two botanicals are further investigated *in vivo* and phytochemical analyses done to establish their suitability in protecting yam tubers against rot fungi.

Conflict of interests

The author declares that there is no conflict of interest.

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REFERENCES

Aboagye-Nuamah F, Offei SK, Cornelius EW, Bancroft RD (2005). Severity of spoilage storage rots of white yam (*Dioscorea rotundata* Poir.) Annals Appl. Bio. 147(2):183-190

Adesiyan SO, Odihirin RA (1975). Histopathology studies of the yam tuber (*Dioscorea rotundata* Poir) infected with *Scutellonena bradys* (Steiner & Hettew). Int. Biodet. Bull. 11:48-55.

Amusa NA, Adegbite AA, Muhammed S, Baiyewu RA (2003). Yam diseases and their management in Nigeria. Afr. J. Biotechnol. 2(12): 497- 502.

Barnett HL, Hunter BB (1972). Illustrated Genera of Imperfect Fungi. 3rd Edition. Burg. Pub. Co.

Ezeibekwe IO, Ibe AE (2010). Fungal organisms associated with yam (*Dioscorea rotundata*, Poir) rot at Owerri, Imo State of Nigeria. Jr. Mol. Gen. 2(1). 1-5.

Foster S, Yue CX (1992). Herbal Emissaries: Bringing Chinese Herbs to the West. Rochester, Vt: Healing Arts Press.

IITA (1993). Crops Improvement Division/ Tuber root Improvement Program Archival Reports (1989-1993). Part III yam. Ibadan, Nigeria. pp. 20-85.

Kuhn PJ, Hargreaves JA (1987). Antifungal substances from herbaceous plants. In: FG Peggs and AG Ayes (eds). Fungal infection of plants. Symp. British Mycol. Soc. CambridgeUni. Press. pp. 48-129.

Marthur SB, Kongsdal O (2003). Common Laboratory Seed Health Testing Methods for Detecting Fungi, 2nd Edition. Int. Seed Testing Association. Switzerland.

Nyadanu D, Dapaah H, Agyekum AD (2014). Resistance to post-harvest microbial rot in yam: Integration of genotype and storage methods. African Crop Sci. Jr. 22(2):89-95

Oduro KA, Damptey HB, Adimora LO (1991). Evaluation of some traditional control measures for diseases of stored white yam, *Dioscorea rotundata* Poir variety Gboko. Ghana J. Sci. 31-36:3-10.

Okigbo RN, Ikediugwu FEO (2002). Evaluation of water losses in different regions of yam (*Dioscorea* spp.) tuber in storage. Nig. J. Exp. Appl Biol. 3. 320.

Okigbo RN, Nmeka IA (2005). Control of yam tuber rot with leaf extracts of *Xylopia aethiopica* and *Zingiber officinale*. Dept. Microbiol., Michael Okpara Univ. Agric. Textbook, Umudike. pp. 806.

Pandey DK, Tripathi NN, Tripathi RO, Dixit SN (1982). Fungitoxic and Phytotoxic properties of essential oil of *Phylis sauvolensis*. Pfkrankh. Pfschutz. 89:344-346.


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