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Molecular Characterization of Canine Rabies Virus Strains Circulating in Mali

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Abstract: Rabies are one of the major zoonoses in Mali. The control of this disease is based on the use of effective diagnostic techniques, the management of human cases after exposure and medical prophylaxis of animals. Diagnosis is usually made by detection of viral antigen by direct immunofluorescence (IF). Due to the increasing variability of the genus Lyssavirus observed, it was found necessary to use a complementary technique, namely reverse transcription in polymerase chain reaction (RT-PCR). The objective of the present study was to assess the diagnostic performance of IFD and RT-PCR for the diagnosis of animal rabies in Mali with a view to characterizing the different strains identified at the molecular level (gene of rabies virus nucleoprotein N). Thus, the analysis of forty (40) brain samples from dogs suspected of having rabies, revealed 36 positive cases for IFD, i.e. 90% and 33 for RT-PCR, i.e. 82.5.% and a concordance rate of 82.5%. Analysis of the segment of the gene of nucleoprotein (N) showed that the rabies viruses circulating in Mali belong to the Africa 2 lineage (Africa 2). Statistical analyzes showed the existence of a significant difference ($P=0.001<0.005$) between the performance of the two techniques. The present study shows the usefulness of the complementarity of the two tests as an alternative for confirming the diagnosis of rabies.

Keywords: Canine Rabies, Comparative Diagnosis, IFD, RT-PCR, Genotype

1. Introduction

Rabies is a viral zoonosis that affects all mammals. It is caused by a virus of the genus lyssavirus of the Rhabdoviridae family. It is usually transmitted to humans as a result of biting, scratching or licking a wound by a rabid animal [1].

It also causes a significant loss of livestock with 11,500 cattle deaths reported each year in Africa and 21,150 deaths in Asia. It also causes around 60,000 reported deaths in humans each year and remains a public health problem in many countries around the world [2-4].

In Africa cases have been recorded in almost all areas. In addition, 248 cases of human rabies were reported from 2015 to 2020 in Mali [5].

Rabid dogs are responsible for the majority of human cases in Bamako. It is most prevalent in underserved communities that have limited access to the health system (human and animal). In addition, there is a problem of accessibility to the diagnostic test throughout the territory of Mali and insufficient knowledge about the burden of the disease in Mali. Indeed, the differential diagnosis with other viral encephalitis of different etiology is difficult, if not impossible. Under these conditions, only the laboratory examination can make a definite diagnosis [6-8].

Due to the increasing variability of the genus lyssavirus observed, the development of diagnosis and differentiation is

also proving difficult. To date 12 species of lyssavirus and three species not yet classified have been identified. Histologically, the signature of rabies infection is the presence of acidophilic cytoplasmic inclusions in certain nerve cells (Negri bodies) [9, 10].

Prompt confirmation of the diagnosis of rabies is extremely important in order to prevent the spread of infection and to initiate timely post-exposure prophylaxis [11].

Today, the IFD which is considered as the "gold standard" in the diagnosis of rabies in most diagnostic laboratories has shown its limits due to the absence of formation of the "body of Negri" in certain strains viral. Hence the need to combine an additional diagnostic test for the final confirmation of suspected cases of rabies. This is how we decided to carry out the present study in order to evaluate the performance of IFD and RT-PCR in the diagnosis of rabies, for the implementation of a molecular diagnosis in addition to the IFD [10].

2. Material and Methods

2.1. Study Site

The present study, a comparative diagnosis was carried out at the Central Veterinary Laboratory (LCV) of Bamako, in laboratories specialize in molecular biology and rabies diagnosis. The molecular identification isolated strains was carried out by the FAO reference laboratory (Padua in Italy).

2.2. Collecting Samples

Forty brains of dogs received at the LCV and suspected of being infected with rabies made up the samples. They come from various horizons of the country, because of the fact that the LCV is the only laboratory in Mali with the ability to diagnosis animal rabies.

2.3. Diagnostic Techniques

2.3.1. Direct Immunofluorescence

This is the gold standard for diagnosing rabies. Each dog's head was cut open with a sterile blade, forceps and pair of scissors. Two appositions were made with a spoon on a blade, one from the horn of Ammon and the other from the medulla oblongata, if possible, otherwise the cerebral cortex.

After drying, the slide was soaked in a bath containing a cold for 30 minutes. Each series of diagnostic tests includes a positive control and a negative control, the purpose of which is to verify the activity of the conjugate used.

After drying in the open air, 25 µl of conjugates were deposited on each slide. The slides were incubated in a humid chamber for 15 minutes. The last fixation step consists of incubating the slides at 37°C in a humid atmosphere for 30 minutes.

The slide was then washed with PBS (PH=7) for 5 min to remove the conjugate, then summarily dried.

Evans blue has been added to the 1/2000 conjugate for ease of reading. It was done in the dark with an immunofluorescence microscope by adding buffered glycerin (immersion oil).

The sample was considered positive when a fluorescent inclusion of green or yellow-green color appeared, clearly brilliant, of various shapes and sizes.

2.3.2. Method for Detecting the N Gene of Rabies Virus by RT-PCR

About 2 grams of biological material (brain) were triturated and homogenized in 500 µl of sterile PBS. This solution constitutes the viral suspension. To inactivate the rabies virus, 100 µl of this suspension were added to 400 µl of Lysis buffer (396 µl of RLT buffer + 4 µl of β mercapto-ethanol) in a 1.5 ml tube. The tube is well vortexed. RNA was fixed on the RNeasy column by adding 500 µl of 70% ethanol to the virus suspension.

Then 700 µl of this mixture, including the precipitates, were placed in the RNeasy column placed in a 2 ml collection tube. The tube was centrifuged for 15 seconds at 4,000 rpm. The effluent in the collector is discarded. The remaining volume (300 µl) was also deposited on the RNeasy column and centrifuged as described previously. The effluent is discarded each time. To remove all the impurities on the RNeasy column, two successive washes were carried out:

1. The first washing was carried out with 700 µl of RW1 buffer and centrifuged at 4,000 rpm for 15 seconds, then the RNeasy column was transferred to a new 2 ml collection tube.
2. For the second wash, 500 µl of RPE buffer were placed on the RNeasy column and centrifuged at 4,000 rpm for 15 seconds. To thoroughly dry the membrane of the RNeasy column, 500 µl of RPE buffer were further added and centrifuged at 12,000 rpm for 2 minutes.

The RNeasy column was transferred to a new 1.5 ml collection tube. In each tube, 50 µl of RNase-free water was deposited directly on the column membrane. The tube left to stand for 2 to 5 minutes was centrifuged at 8,000 rpm for 1 minute.

The RNAs thus extracted were stored at -20°C before immediate amplification by the technique of One Step RT-PCR.

Amplification of the N gene (Nucleoprotein) was done by the technique of One-Step RT-PCR. The reaction mixture (Master mix) was prepared in a 1.5 ml tube, according to the proportions indicated in Table 1.

The mix was distributed at a rate of 20 µl per PCR tube and 5 µl of extracted RNA were added to each PCR tube in the corresponding strip. The primer sequences used are listed in Table 2.

The strip PCR tubes were placed in the Morel GeneAmp® PCR System 9700 "Applied Biosystem thermocycler" and amplification was started according to the schedule below in Table 3.

Table 1. *Concentration and volume of reagents used.*

Name of reagents /initial concentration	Final concentration	Volume for 1 reaction (µl)	Volume for 15 reactions (µl)
RNase free water	/	11,50	172,5l
PCR Buffer 5X	1X	5	75
dNTPs mix 10 mM	0,4 mM	1	15
Amorce Rab For pyro 10 µm	0,4 µm	1	15
Amorce Rab Rev pyro 10 µm	0,4 µm	1	15
One Step RT-PCR Enzyme Mix		0,5	7,5
Volume without RNA		20	300
ARN extract		5	
Final reaction volume		25	

Table 2. *Primers used.*

Name of reactions	Codes de la compagnie	Conservation condition
Amorce Sens: RabForPyro 5' -AAC ACY YCT ACA ATG GA- 3'	Eurofins	-20°C (+2-10)°C
Amorce anti sens: RabRevPyro 5'-TCC AAT TNG CAC ACA TTT TGT G- 3' 5'-TCC ART TAG CGC ACA TYT TAT G- 3' 5'-TCC AGT TGG CRC ACA TCT TRT G- 3'	Eurofins	-20°C (+2-10)°C
Qiagen One Step RT-PCR kit	Qiagen Cod. 210212	-20°C (+2-10)°C
Rnase Inhibitor	Promega cod. n° N2611	-20°C (+2 -10)°C

Table 3. *Amplification program.*

Reverse Transcription	Inactivation de la RT-PCR et activation de la Taq polymérase	PCR			Élongation finale	Conservation
		Dénaturation	Hybridation	Élongation		
50°C	94°C	94°C	52°C	72°C	72°C	4°C
30 min	15 min	30 secondes	30 secondes	40 secondes	5 min	∞
1 cycle		35 cycles			1 cycle	

All the steps of cDNA synthesis as well as DNA amplification were carried out at the thermocycler according to the program in Table 3.

The amplicons (amplified DNA fragment) were deposited in a 2% agarose gel. Migration was done in 1X TAE buffer. Two (2) µl of the loading buffer was added to the intercalating agent (the red gel) and then mixed with 8 µl of amplicon (PCR product) on parafilm. This mixture (10 µl) was placed in all the corresponding wells of the gel (except the first well). Then, 5 µl of the molecular weight marker (DNA ladder) was placed in the first well to assess the size of the PCR products. The migration was carried out at a voltage of 100 volts for 45 minutes.

After electrophoresis, the gels were visualized using the Gel Box under ultra violet light (254 nm to 365 nm). The gel was read by the Vision-Capt software.

2.3.3. Sequencing of Identified Rabies Virus Strains

The PCR products were partially sequenced for the gene the nucleoprotein (N) according to the protocol of the QIAGEN kit. The sequences of the amplified fragments were made by the Padua laboratory in Italy (FAO National Reference Center for rabies).

3. Results and Discussion

The results obtained concerning the two tests are recorded in Tables 4 and 5. The bands obtained by the RT-PCR are in figure 1. Table 6 shows the areas of origin of the samples and the subgroups identified in the line. Africa 2.

Table 4. *Summary of analyzes of variance for virus identification tests, specimens and test-specimen interaction.*

Variables	DDL	Moyenne des carrés
		Détection des virus
Tests	1	0.10810193***
Samples	39	0.15140592***
Tests*Samples	39	0.08565971***

The analysis of the data in table 4 showed that the tests used detect rabies virus differently. In addition viruses are detected differently from sample to sample. Finally, the detection of the virus by any of the tets used varies depending on the sample used.

Table 5. *Test efficiency.*

Tests	Number of observation	Average virus presence
IFD	80	0,6238a
RT-PCR	80	0,5718b

Analysis of the data in table 5 showed that the IFD test has a higher efficiency in detecting rabies virus than RT-PCR

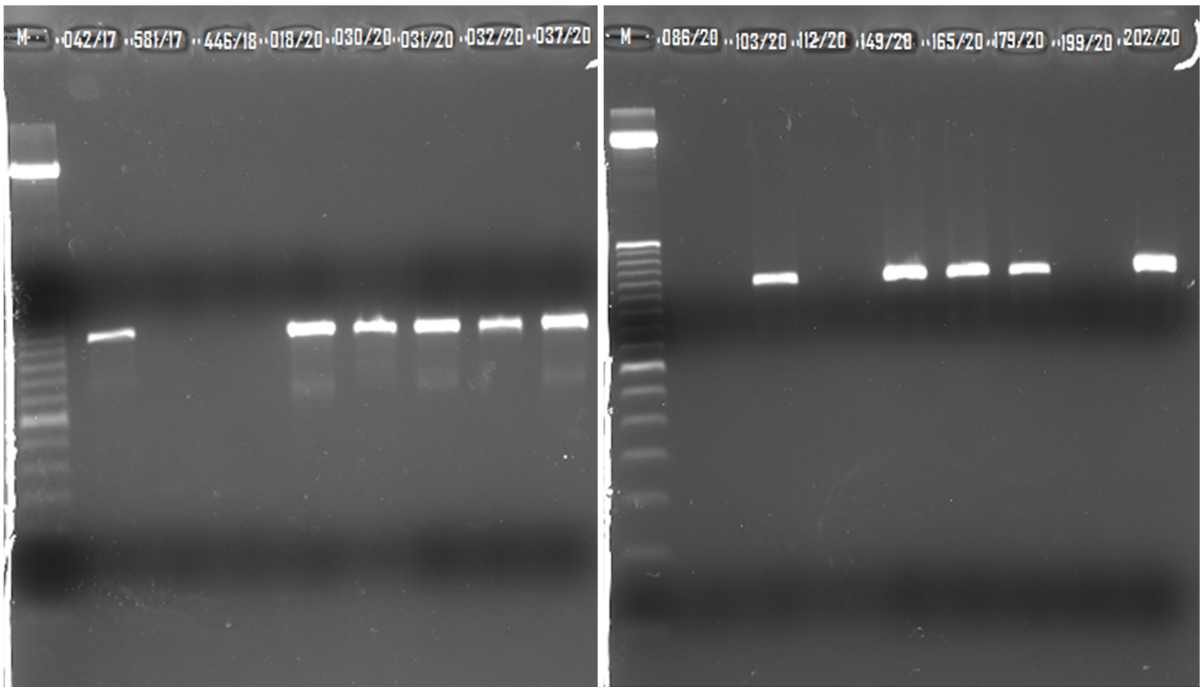

Figure 1. *Results of the detection of bands in PCR.*

Table 6. *Presentation of samples showing the areas of provenance, the cycle threshold (Ct) and the subgroup of the Africa 2 line to which the sample belongs.*

Samples ID number	Area of origin	Quantitative RT-PCR C_t	Africa 2 lineage subgroup
018/20	Koury	29.5	G
042/17	Bamako	30.7	G
051/19	Kati	38.6	G
086/20	San	22.0	G
112/20	Douentza	25.0	G
149/20	Kati	34.3	G
179/20	Kolondieba	22.9	H
199/20	Nioro	28.7	G
202/20	Douentza	36.4	Nd
204/20	Kati	24.4	G
249/20	Ségou	Nd	Nd
315/20	Mopti	38.5	G
316/20	Koulikoro	27.3	G
331/20	Bamako	24.4	G
478/20	Kolondieba	28.0	H
883/20	Kati	28.9	H
504/20	Kati	30.0	G
505/20	Yorosso	26.2	G
540/20	Kati	27.2	G
825/19	Bamako	Nd	Nd
883/19	Kati	26.3	G

Ct: cycle threshold; the samples are considered positive if the Ct<36, doubtful if the 36>Ct<45 and negative if the Ct>45. Nd: not available.

Controlling rabies is a complex task due to its multidimensionality. Samples received at the LCV showed that dogs are the first victims and source of rabies, as 100% of these samples are of canine origin. This situation is comparable to those described by [12, 13]. These authors had found, respectively, that 97.19% and 84% of the samples received were composed of dogs suspected of rabies. However, this situation is different from the results observed in Côte d'Ivoire by [14] where dog brains represented 74.3% of samples received in the laboratory.

Traditionally, the diagnosis of animal rabies is made by the detection of the viral antigen of the rabies virus by direct immunofluorescence (detection of the Negri body) in the brain tissues of the suspect animal, but the bodies of Negri cannot be demonstrated in all strains of rabies virus [15].

Of the 40 samples examined, 36 tested positive for IFD (90%) and 33 (82.5%) for RT-PCR. In an inter-laboratory test as a tool to increase animal rabies diagnostic capabilities in veterinary laboratories in sub-Saharan Africa, RT-PCR had a sensitivity of 96.7% and specificity of 100%, both higher than those obtained by [16] with IFD (89.2% and 86.2%, respectively). Manisha Biswal and al showed 100% sensitivity and specificity to RT-PCR, compared to 83.3% to IFD. On the other hand, in the Central African

Republic and more particularly in Bangui, a study carried out by Mangué J as part of his thesis found 100% of samples positive for IFD and 93% for RT-PCR [11, 17].

A study conducted by Paola and al found one RT-PCR negative sample that tested positive for IFD, and 8 RT-PCR positive samples were not confirmed by IFD, but all were confirmed as a rabies virus of the Rhabdoviridae family (RABV) by both pyrosequencing and conventional sequencing. The RT-PCR was applied to these 8 samples, confirming that 4 of them were positive [18].

Our results are still almost similar to those obtained by Dao and al in Bamako 98.35%, Selly and al in Ivory Coast 90%. These high prevalences of dogs rabies in Bamako (98.35%) and in Bangui (97.76%) confirm our results (97.76%) [6, 12-14].

Analysis of the segment of the gene of nucleoprotein (N) showed that the rabies viruses circulating in Mali belong to the Africa 2 line, circulating in the canine population of Central and West Africa [19, 20].

Phylogeny (figure 3) reveals more than any other method the diversity of the viral population constituting the genus Lyssavirus and that each phylogenetically specified genotype has its own characteristics concerning its distribution in the world and its host spectrum [21].

Phylogenetic analysis of partial sequences for the N gene showed that the *lyssaviruses* circulating in Mali belong to the Africa 2 lineage (Figure 3). On the phylogenetic tree, viruses from this study are marked in red. Other viruses originating in Mali and available from the public database (Genbank) are indicated in orange. Analysis of the nucleoprotein (N) gene identified two subgroups which are G and H with a slight dominance of the G subgroup (Figure 3).

Rabies viruses from Mali cluster with viruses circulating in neighboring countries (Figure 2) such as Burkina Faso, Côte d'Ivoire, Guinea-Conakry, Liberia, Mauritania and Senegal. The viruses in this study belong to groups G and H of the Africa 2 line (figure 3) and therefore the presence of viruses belonging to group F (virus identified towards the north of Mali at the Niger border) as previously observed by Traoré A and al was not detected. These strains differ little within a line. Further study will allow the extension of virus strains to be followed and could help determine their origin [20].

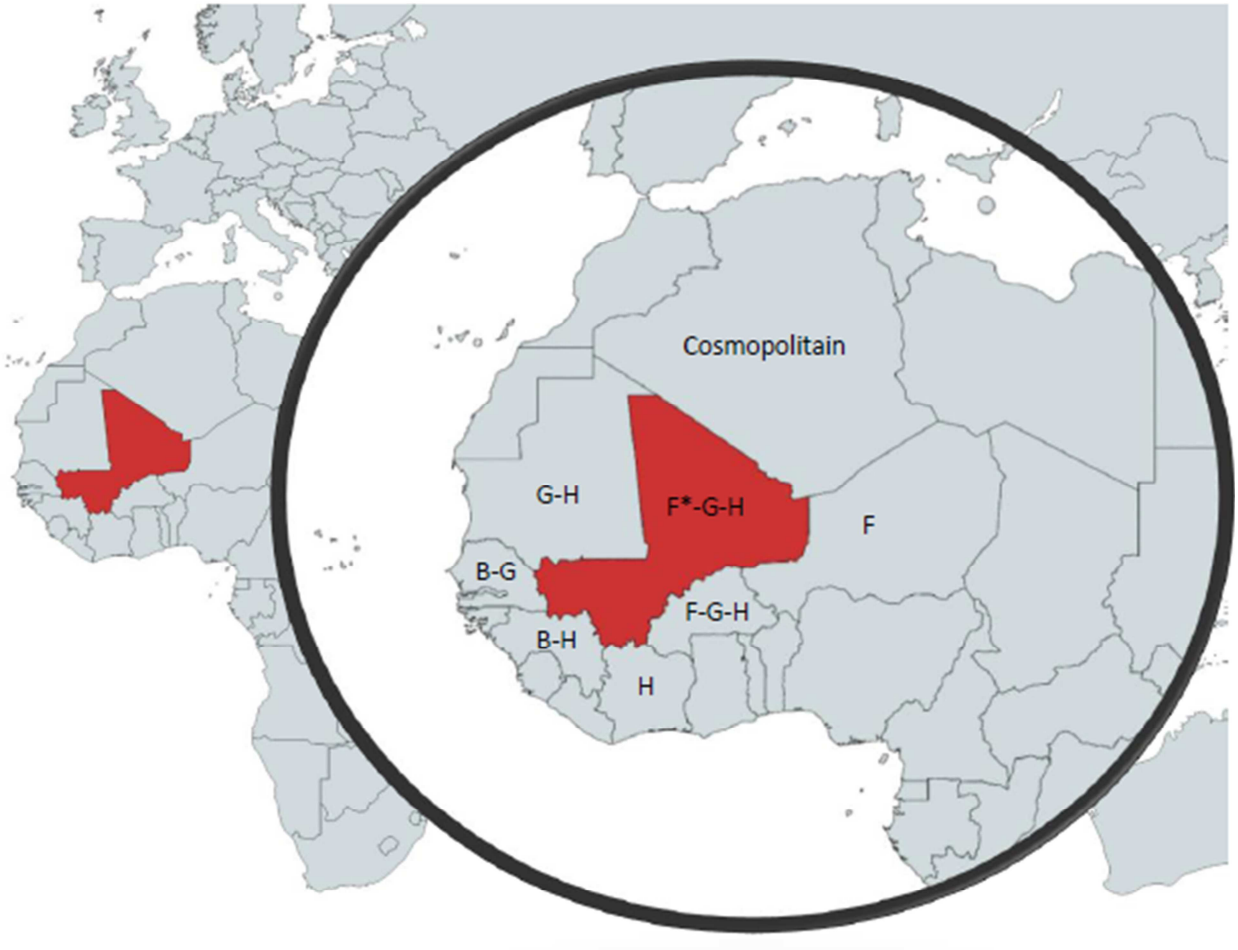

Figure 2. *Africa 2 rabies virus identified in Mali and neighboring countries.*

Figure 3. *Phylogenetic tree obtained after sequencing the RNA of rabies viruses.*

All viruses circulating in Senegal, Liberia, Ivory Coast and Mauritania have shown a strong similarity to those in Mali. Bootstrap values greater than 70% are shown next to the nodes.

4. Conclusion

This study evaluated the performance of IFD and RT-PCR for the diagnosis of rabies. It established that there is a significant difference between the performance of the two techniques in the context of the diagnosis of rabies. It is necessary to use RT-PCR as a complementary test to IFD for the diagnosis of rabies.

She highlighted in Mali two subgroups (G and H) of the Africa 2 lineage.

The results of this study will contribute to improve knowledge on the epidemiology of rabies for effective control of this infection.

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