Resistance of Trypanosoma congolense forest isolates to isometamedium Chloride and diminazene aceturate in West Democratic Republic of Congo

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ABSTRACT

Resistance to trypanocides is increasing rapidly in sub-Saharan African countries where African animal trypanosomiasis occurs. An investigation was carried out over a four-year period to assess trypanosome resistance to trypanocides in the western Democratic Republic of Congo (DRC) using the standard mouse test. Eight strains of *Trypanosoma congolense* Forest, two from Kimwenza and one from N'Djili Brasseries in the City of Kinshasa Province, three from Mbanza Ngungu in the Province of Central Kongo and two from Mushie in the Province of Mayi Ndombe, were isolated from pigs and cattle. Following the amplification in mice, 10^5trypanosomes of each strain were inoculated in three groups of six NMRI mice. Twenty-four hours later, the first group was treated with 1g of isometamidium chloride (ISM)/kg BW/mouse, the second group was treated with 20mg of diminazene aceturate (DA)/kg

BW/mouse while the third group was left untreated and served as control. All these mice were followed up for the development of parasitemia for a period of two months. At the end of the experiment, only the N'Djili Brasseries strain was resistant to ISM at a dose of 1 mg / kg bw. When multidose test was applied, no resistance was observed in groups of mice treated with high doses (from 2 to 20g ISM/kg bw/mouse). This study shows for the first time the occurrence of trypanosome resistance strains to ISM in DRC. The DRC becomes the 20th sub-Saharan country of the chemoresistance of trypanosomes to trypanocides has been reported.

Key Word: Resistance, *Trypanosoma congolense* Forest, Isometamidium chloride, Diminazene aceturate, mice, DRC.

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INTRODUCTION

In sub-Saharan Africa, African animal trypanosomiasis (AAT) remains widespread (Pangui, 2001). About 60 million cattle, 100 million small ruminants and other animal species are threatened in nearly 10 million km2 in 37 countries in this part of Africa (Trial et al., 1985, Geert et al., 2001), with significant annual meat losses estimated at nearly US $ 5 billion (Mortelmans, 1986). In these areas, disease control is either managed by vector or parasite control or by combining the two. However, in poor rural communities, which are most affected by the disease, control is mainly based on the use of trypanocides (Delespaux and al., 2008).

The main drugs used by breeders are isometamidium chloride (ISM), which has curative and prophylactic effects, and diminazene aceturate (DA), which has only curative effect. These drugs have been in use for over half a century. Geerts and Holmes (1998) estimated that 35 million doses of trypanocides are administered each year in sub-Saharan Africa. Despite the high usage of these veterinary trypanocides, the interest of the pharmaceutical industry to invest in research to develop new products remains low, leaving farmers to rely on existing drugs.

Due to the privatization of veterinary services in most parts of Africa, farmers have easy access to these trypanocides and this has resulted in widespread misuse and under-dosing of drugs, actions that have been blamed for the emergence of trypanocidal drug resistance (Eisler and al., 2000, Van den

Bossche and al., 2000, Geerts and al., 2001, Delespaux et al., 2002, Talaki et al., 2009). Trypanosome resistance to trypanocides has been reported in 21 countries in sub-Saharan Africa (Talaki and al., 2013) including five countries bordering the Democratic Republic of the Congo (DRC). However, the evaluation of trypanocidal drug resistance in DRC has so far not been conducted although trypanocides has been circulating in the country for several decades. Thus the present study was initiated to evaluate the level of trypanosome resistance to trypanocides in western DRC using the standardized mouse test (SMT).

MATERIAL AND METHODS

Study area

This study took place for four years from 2013 to 2016 in the Mushie ranch focus (Latitude 02 ° 09 'to 2 ° 95' and Longitude 16 ° 37 'E) in Mayi Ndombe Province, in Kimwenza (Latitude: 4 ° 27 '33' 'S and Longitude: 15 ° 17' 20 " E) and in N'Djili Brasseries (Latitude: 04 ° 48 '08' 'S and Longitude: 15 ° 30' 43 " E) in the Province of Kinshasa and in Mbanza Ngungu (Latitude: 05 ° 27 '127' 'S and Longitude: 014 ° 97' 173 " E) in Central Kongo Province in Western DRC. Molecular analysis and Detection of trypansome resistant strains conducted at the Veterinary Laboratory in Kinshasa and the National Institute of Biomedical Research (INRB), respectively.

Trypanosome isolation in the field

Trypanosomes were isolated from cattle and pigs that were positive through parasitological diagnosis with the buffy-coat method. A volume of 200 µl of infected blood was inoculated intraperitoneally in two NMRI adult mice weighing 25-30 g. Mice were followed up three times a week for the development of parasitemia for a two-month period. The monitoring consisted in microscopic examinations for the presence of trypanosomes from a drop of blood collected from the tail of each mouse. Once a mouse was tested positive and the parasitemia reached a concentration around 10^7 trypanosomes/ml by the Matching method (Herbert and Lumsden, 1976), the animal was euthanized to collect blood from the heart. The blood collected (about 1ml) was mixed with a cryoprotectant (Triladyl) and stored in the liquid nitrogen as stabilate until further use.

Molecular characterization of trypanosome isolates collected from the field

Following the detection of trypanosomes by the buffy-coat method, the infected blood was spotted onto a filter paper and dried at room temperature. Molecular characterization was carried out at the Central Veterinary Laboratory using the PCR technique developed by Geysen and al. (2003) for the characterization of trypanosomes up to genus level and by Desquesnes and al. (2001) for the species characterization.

Testing for trypanosome resistance in mice

The SMT was performed according to the protocol described by Eisler and al. (2001). NMRI adult mice weighing 25 to 30 g were used. For each isolate, three groups of six mice were sub-inoculated intraperitoneally with 10^5 trypanosomes collected from parasitaemic mice that were previously inoculated using the stabilates kept at the liquid nitrogen. Twenty-four hours later, two of these groups were treated with 1mg of ISM /kg bw/mouse and 20mg of DA/kg bw/mouse, respectively. The third group was left untreated and served as the control group. All these mice were followed for a period of two months for the development of parasitemia. Data on parasitemia and PCV were collected three times a weeks for the first two weeks and twice a week from the third week until the end of the experiment (Masumu and al., 2006).

Following this protocol, a trypanosome isolate was considered resistant if more than one of the six mice relapse after treatment and the test was valid if at least five of the six control mice develop parasitaemia after inoculation of the isolate; otherwise the test was completely repeated. For a resistant strain, a multi-dose test was performed using five different doses ranging from 2 to 20 mg / kg (2, 5, 10, 15 and 20 mg/kg) for ISM.

Investigation on the sale and use of trypanocides

Questionnaires were submitted to veterinary drug merchants in Kinshasa and to pig breeders in the area where drug resistance was observed to obtain information about trypanocidal use practices and to assess their knowledge on AAT.

RESULTS

Molecular characterization of trypanosome isolates

Following the 18S primers used to characterize the eight isolates that were collected from cattle and pigs in the study areas, the expected band was produced successfully suggesting that all the isolates belonged to the *Trypanosoma* spp. To further characterize these isolates at species level, the ITS technique used revealed the presence of *Trypanosoma congolense* West African Riverine and Forest (WARF) in all the eight isolates.

Mouse test for the detection of ISM and DA resistance

In total, 48 groups of six NMRI mice were infected with 1mg/kg bw/mouse of ISM (24 groups) and 20 mg/kg bw/mouse of DA (24 groups) making a total of 288 mice being followed up. For each drug, three groups were infected with each of the eight isolates among which one group was treated, 24h later, with ISM, one with DA while the third was left untreated. After 60 days of monitoring of all the groups of mice, relapses were observed in two of the six mice infected with the isolate collected from a pig in N'djili Brasseries/Kinshasa and subsequently treated with ISM. All the mice infected with the remaining seven isolates were cured following the treatment with ISM. Similarly none of the mice infected with the eight isolates and further treated with DA relapsed, apart from one *T. congolense* isolate collected from Kimwenza with a single mouse relapsed (Table I).

The *T. congolense* WARF isolate that was resistant to 1mg of ISM/kg bw/mouse was further tested with higher doses (2, 5, 10, 15 and 20 mg/kg bw/mouse). As shown in Table II, a single mouse in the group treated with 2mg/kg bw/mouse relapsed.

Table I. Standard Mouse test for the detection of DA and ISM resistance in eight isolates of *T. congolense* WARF in western DRC

Isolate	Site	Host	Number of mice that relapsed after treatment with	
			DA (20 mg/kg bw)	ISM (1 mg/ kg bw)
K01	Kimwenza	Pig	1/6	0/6

K02	Kimwenza	Pig	0/6	0/6
Mb01	Mbanza Ngungu	Pig	0/6	0/6
Mb02	Mbanza Ngungu	Pig	0/6	0/6
Mb03	Mbanza Ngungu	Pig	0/6	0/6
Mu01	Mushie	Cattle	0/6	0/6
Mu02	Mushie	Cattle	0/6	0/6
N01	N'Djili Brasseries	Pig	0/6	2/6

Table II. Multidose testing of ISM resistant *T. congolense* WARF isolates

Number of relapses following treatment with isometamidium chloride at				
2 mg/Kg bw	5 mg/Kg bw	10 mg/Kg bw	15 mg/Kg bw	20 mg/Kg bw
1/6	0/6	0/6	0/6	0/6

Socio-professional characteristics of pig breeders in N'djili Brasseries/Kinshasa

The survey on the use of trypanocides involved 100 pig breeders from N'djili Brasseries among which 86% were men. The majority of the respondents had the secondary (71%) or the university (22%)

level. Most of them were farmers (67%) or traders (14%). More than half of the respondents had less than 10 years of experience of pig breeding with the majority having less than 20 years. In most cases farmers kept only pigs at their moesteads although some of them conducted mixed farming system with cattle (1%), goats (7%), sheep (2%) and rabbits (6%). Dogs and cats were found in 37 and 19% of homesteads respectively (Table III).

Table III. Socio-professional characteristics of pig breeders in N'djili Brasseries/Kinshasa

Variable	Number	Proportion
Gender (n= 100)		
Male	86	86%
Female	14	14%
Level of instruction (n = 100)		
None	1	1%
Primairy	6	6%
Secondary	71	71%
University	22	22%
Profession (n= 100)		
Farmer	67	67%
Veterinarian	2	2%
State officier	4	4%
Trader	14	14%
Teacher	7	7%
House builder	2	2%
Physician	2	2%

Electrician	2	2%
Experience in livestock farming	**n=100**	
<10 years	56	56%
10-20 years	27	27%
21-30 years	8	8%
> 30 years	9	9%
Animal species present in the exploitation	**n=100**	
Pigs	100	100%
Cattle	1	1%
Goats	7	7%
Sheep	2	2%
Rabbits	6	6%
Dogs	37	37%
Cats	19	19%

Knowledge, attitude and practices of pig breeders related to trypanosomiasis

The evaluation of the knowledge of trypanosomiasis among pig breeders of N'Djili Brasseries showed that 87% of them had already heard about AAT, 75% knew it was transmitted by tsetse flies but only 15% of the respondents knew it was caused by a parasite. About the treatment, 86% of the respondents said they were aware of the existence of the treatment.

Table IV. Knowledge, attitude and practices of pig breeders related to trypanosomiasis

Variable	Number	Proportion

Knowledge of the disease	***n*=100**	
Yes	87	87%
No	13	13%
Knowledge of the parasite	***n*= 100**	
Yes	15	15%
No	85	85%
Knowledge of the vector	***n*=100**	
Tsetse fly	75	75%
Mosquitoes	6	6%
Sexual contact	3	3%
No information	16	16%
Knowledge of the treatment	***n*=100**	
Yes	86	86%
No	14	14%

Disease management

As shown in Table V, about 80% of farmers were aware of clinical signs of AAT. The disease affected only pigs and 30% of farmers recognized having observed the presence of the disease in pigs at a frequency of one case per year (83.3%) or once per quarter (16.7%). About half of the interviewers (48%) seek assistance from experienced farmers to treat their animals while 46% called for veterinary assistants. Only 6% of respondents used Veterinary Doctors' services although about half of them (46%) were willing to be assisted by them. All of them treated their animals only in case of illness while 96% of them declared systematically checking the expiring date of trypanocides before use. DA was the most used molecule (71%) compared to ISM (9.8%). If the doses administered were generally in the standards, the weight of the animals to be treated was systematically estimated.

Table V. Trypanosomiasis management

Variable	Number	Proportion
Clinical diagnostic	**n=100**	
Edema and anorexia	20	20%
Weight loss and anemia	13	13%
Anorexia and weight loss	41	41%
Agressivity and other signs	1	1%
Edema and weight loss	6	6%
No knowledge of clinical signs	19	19%
Animal health manager	**n=100**	
Farmer	48	48%
Veterinary technicians	46	46%
Veterinary Doctors	6	6%
Willing to be assisted by a Veterinary Doctor for treatment	**n=100**	
Oui	46	46%
Non	54	54%
Trypanocide	**n=100**	
Trypamiduim (isométamidium chloride)	28	29.8%
Samorin (isométamidium chloride)	1	1%
Diminaveto (diminazene aceturate)	5	5.3%

Berenil (diminazene aceturate)	1	1%
Tryponil (diminazene aceturate)	15	15.9%
Veriben (diminazene aceturate)	50	50%
Verification of the expiration date of the trypanocide	**n=100**	
Yes	96	96%
No	4	4%

Trypanocide sales in Kinshasa

The investigation on trypanocide sales in Kinshasa concerned 18 salesmen in veterinary drug stores in the areas of Lemba, Kimbaseke, N'Djili, Kasavubu, Ngaliema, Makala, Gombe, Mont-Ngafula and Masina. Among these 18 respondents, 56% were women. Most of them (72%) were over 30 years old. About 88% of them had a secondary or university level; only 6% had primary or post-graduate level, respectively. Veterinarians, veterinary assistant and agro-veterinarian accounted for 61% of veterinary drug dealers.

Table VI. Socio-professional characteristics of veterinary drugs dealers in Kinshasa

Variable	Number	Proportion
Gender	**N=18**	
Male	8	44%
Female	10	56%
Age	**N=18**	
20 - 25 years	2	11%
26 – 30 years	3	17%
> 30 years	13	72%
Education level	**N=18**	
Primairy	1	6%
Secondary	6	33%
Graduate	10	55%

Post-graduate	1	6%
Profession	**N=18**	
Veterinary technician	4	22%
Veterinarian	4	22%
Agronomist-veterinarian	3	17%
Farmer (crops)	2	11%
Trader	3	17%
Pharmacist	1	5,50%
Farmer (livestock)	1	5,50%

The evaluation of the knowledge of the disease, attitude and practice among veterinary drug dealers in Kinshasa showed that all of them have already heard of AAT and they all sold trypanocides. About 78% of the respondents sold trypanocides more than once a month. The majority (94%) of them make an inventory of their sales that showed that DA is the most sold molecule (89.4%) compared to ISM. The majority (77%) of the drug stores were less than 5 years old. These drugs were sold to three categories of clients: veterinarians (1/3), veterinary assistants (1/3) and farmers (1/3).

Table VI. Knowledge, attitude and practices of veterinary drug dealers in Kinshasa

Variable	Number	Proportion
Knowledge of the disease	**N=18**	**100%**
Yes	18	100%
No	0	0%
Duration of experience	**N=18**	**100%**
> 10 years	3	17%
5-9 years	1	6%
< 5 years	14	77%
Trypanocide sales	**N=18**	
Yes	18	100%
No	0	0%
Trypanocide sale frequency	**N=18**	
> 1X/month	14	78%
Once a month	2	11%
Once a semester	2	11%

Client category	**N=51**	
Veterinary Doctors	16	31,80%
Veterinary technicians	17	33,30%
Farmers	18	35,30%
Trypanocide most sold	**N=47**	
Diminazene aceturate	42	89,40%
Isometamidium chloride	5	10,60%

DISCUSSION

Resistance to trypanocides has been reported in 21 countries in sub-Saharan Africa among which Burkina Faso, Ivory Coast, Ethiopia, Kenya, Nigeria, Uganda, Somalia, Sudan, Tanzania, Chad, Zimbabwe (Peregrine and Mamman, 1993), Central Africa Republic (Finelle and Yvore, 1962), Senegal (Diaite and al., 1995), Zambia (Eisler and al., 2000), Mozambique (Jamal and al., 2005), Cameroon (Mamoudou and al., 2006), Guinea, and Mali (Talaki and al., 2006, Talaki and al., 2009), Ghana (Allegye-Cudjoe and al., 2009), Benin and Togo (Boma, 2012, N'Fiedi, 2012). The results of this study reveals for the first time in the DRC the existence of a *T. congolense* WARF strain resistant to ISM making up the DRC the 22nd country where resistance to trypanocides has been reported in sub-Saharan Africa. Although resistance to these drugs has not been reported in other countries where AAT occurs and where trypanocides have been used for a long period of time, this may probably be under estimated because the situation is ignored in those countries (Geerts and Holmes, 1998). This under estimation of the current situation of trypanocidal dryg resistance throughout the countries where AAT prevails can be supported by the apparent ineffectiveness of trypanocidal treatment as observed in the field in various areas (Diall and al., 2003).

If resistance to trypanocidal drugs has only been found now in the DRC it's also because such surveys have never been undertaken in the country. Indeed assessment of trypanocidal resistance still faces several technical challenges. In vitro assays cannot be applied for some strains of trypanosomes that are very difficult to grow in the culture. The cattle model that is ideal for trypanosomes that infect livestock (*T. congolense*, *T. brucei* or *T. vivax*) has rarely been used in the field because it is very expensive and time consuming. Molecular tests that have been developed for animal drugs including ISM (Delespaux and al. 2005) and DA (Delespaux and al., 2006) have finally been dropped because of inadequate findings in the field. Finally, the mouse model is restricted to trypanosome strains that can develop in mice

(mainly the *T. brucei* group) excluding *T. vivax*, *T. simiae* and some strains of *T. congolense*. Although trypanocide doses in mice cannot be applied in cattle, a good correlation was observed between resistance of *T. congolense* to ISM and DA in both cattle and mice (Bengaly and al., 2002a,b).

Our study made use of the mouse model. In this study, eight isolates of *T. congolense* WARF were tested for their susceptibility to ISM and DA using the standard protocol that was developed by Eisler and al. (2001). After a two-month of monitoring period following infection and treatment, only the isolate from N'Djili Brasseries was resistant to 1mg/kgBW/mouse ISM, which corresponds to a prevalence of 12.5% of the isolates examined. None of the isolates tested was resistant to DA. To test the level of resistance to ISM for this resistant isolate, a multi-dose testing model was used with higher doses. It finally appeared that the resistance observed was limited to 1 mg of ISM/kg bw/mouse. Using the same protocol of mouse model, Mamoudou et al. (2006) found 32.5% of trypanosomes resistance to DA and 27.5% to ISM. Thus our results suggest that the development of trypanocidal drug resistance is only beginning in this study area.

In most studies where *T. congolense* has been characterized for drug resistance, emphasis was laid on the Savannah group. This is because the WARF group is very difficult to grow in mice. In addition, the virulence profile of *T. congolense* WARF is low in cattle (Bengally et al. 2002a) limiting its prevalence in these animals. In the present study, eight *T. congolense* WARF isolates were collected among which only two were from cattle while the remaining six isolates originated from pigs. The low virulence of this strain in cattle can thus explain the susceptibility of the cattle-derived isolates to the drugs used since it can be assumed that animals are rarely treated when they are infected with such low virulent strains. Although the virulence profile of *T. congolense* WARF is not well known in pigs it seems that the imported breeds of pig used mainly in Kinshasa are susceptible to the trypanosomes that circulate in these areas. This can be supported by the fact that, in this area, most pig breeders know the disease, its clinical signs and treatment. Most pig breeders have also declared to have at least one case of trypanosomiasis a year and most of them treat their animals themselves although they are willing to hire the service of Veterinary Doctors that is considered too expensive for small farmers.

The results of this survey is encouraging to the Congolese farming industries. Indeed livestock systems in some countries where trypanosomiasis occurs is threatened by the development of drug resistance in the field. Since the development of ISM in the mid-sixties and the subsequent development of resistance in various countries in sub-Saharan Africa, no other drug has been put on the market. Currently only ISM

and DA remain the two drugs that are in use in the field against AAT. This suggests that one has to be cautious with the way the two drugs are used because once the resistance develops against both drugs in a given area trypanosomiais control will become hypothetical. This is the case in several countries where drug resistance has developed to both drugs. In DRC however, it can be assumed that the way is still very long before such situation can develop in the field. Our findings support this since factors that predispose to the development of drug resistance in the field were rarely found in the study area. Our data showed that most farmers verify the expiring date before administering trypanocide drugs to their animals. They also respect the dosage although the weight of animal to be treated is always estimated. In this study area, farmers use production-oriented strategy whereby only sick animals are treated thus, reducing the number of treatments per animal/year.

It is a bit surprising that while most farmers prefer DA rather than ISM, resistance has been found here with the later rather than the former product. This finding can be explained by the fact that ISM lasts longer in the animal after treatment while DA is quickly metabolized and removed from the blood circulation. The long remanence of ISM (Geerts and Holmes, 1998) and long interval between two treatments (Peregrine and al., 1991) have previously been suspected as causes of drug resistance development in the field.

CONCLUSION

Our study revealed for the first time the existence, in DRC, of resistance to ISM of a *T. congolense* WARF isolate collected from a pig. For now, the phenomenon seems to be very limited since no resistance has been found in other parts of the countries despite the high number of animals that were sampled. This resistance seems also to be limited to ISM since no evidence of resistance to DA has been observed in the field. In other to avoid possible extension of drug resistance in other parts of the country, it becomes imperative to ensure the detection and continuous monitoring of animal trypanosomiasis and drug resistance in the field. Strategies that need to be adopted should emphasize on the strengthening of the capacity of livestock farmers to better use the two trypanocides that exist in the market. Veterinary services and researchers on animal diseases should also be sensitized on various techniques that address the issues of drug resistance in the field.

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