

# Properties and Antibiotic Susceptibility of *Bacillus anthracis* Isolates from Humans, Cattle and Tabanids, and Evaluation of Tabanid as Mechanical Vector of Anthrax in the Republic of Chad

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## Abstract

Anthrax is a zoonose caused by the organism *Bacillus anthracis*. Anthrax affects mainly animals, particularly cattle, but also humans. The economic loss caused by Anthrax in the Republic of Chad are very important. In this study, the potential role of tabanids in anthrax transmission has been evaluated. Tabanids were collected in infested areas of the Chari-Baguirmi Province in Chad and examined using standard bacteriological and biochemical methods. Hundreds of anthrax bacilli were isolated: in this, about 89% of the 1499 tabanids examined were contaminated by anthrax organisms. *B. anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%). Anthrax Bacilli were also isolated from cattle and humans, including nine cutaneous cases associated with tabanids bites. All *B. anthracis* isolates displayed similar biological properties whatever their origin. They were non motile, sensitive to penicillin, non hemolytic and fully virulent as they can elicit the disease in experimental animals. All but one biochemical character were identical out of the 49 tested. Isolates were sensitive to meticcillin, tetracycline, nitrofurantoin, piperacillin, oxytetracycline, sulphathiazol, benzathien-penicillin and chloramphenicol. Resistance was found against polymixin, fusidic acid, clindomycin and sisomicin. Given together, the results suggested that tabanids could be an important vector of Anthrax in Chad.

**Keywords:** Chad, Anthrax, *B. Anthracis*, Biology, Biochemistry, Antibiotics, Tabanids, Vector, Mechanical Transmission.

## 1. Introduction

*Bacillus anthracis*, the ethiological agent of anthrax, is a large encapsulated Gram-positive rod. It grows vegetatively within an infected host animal and sporulates when it is exposed to the atmosphere or harsh environments (Hunter (1989), and Turnbull (1990)). Spores show a high degree of physical resistance, and can survive for years in soil (Manchee (1981), and Wilson (1964)). The longevity of the spores in the environment is an important biological factor in the distribution of Anthrax.

In the Chari-Baguirmi Province of Chad, temperature usually higher than 40°C and alternance of heavy rains (from May to October with an average 650 mm) and windy dust and dry weather (from November to April), create optimal conditions for the occurrence of Anthrax outbreaks. Soil covering the infested area has a pH ranging 6,5-7,0 that can sustain the persistence of Anthrax. Moreover, the disease transmission can be further complicated by the hatching period of various flies, mainly tabanids.

The role of insects in the spread of *B. anthracis* during anthrax outbreaks has been reported. Several Hematophagous diptera are able to transmit *B. anthracis* mechanically. Some other diptera have the ability to contaminate different surfaces either with spores or bacilli (Kozel (2007), and Nazil (2005)). Flies may transmit Anthrax from animal to animal or from animals to man (Sen, 1944). Herren (1989) pointed out the importance of biting flies in the epidemiology of Anthrax, included tabanidae and Lipoptena. It has been reported that blood-sucking flies (e.g. Hippobosca and Aedes), as well as flies that do not suck blood (like *Mousca domestica* and Flesh eating flies), could transmit Anthrax (Blackburn, 2010; Fasanella *et al.*, 2010). The mouthparts of tabanids are adapted for both blood-sucking and lapping, and particularly well suited to animal. The labella are retracted to expose the fascile which pierces the skin and lacerates the tissues for pool-feeding. The mandibles move with a scissor-like action and the maxillae move forwards and backwards (Dickerson and Lavoipierre, 1959b). When feeding ceases, the fascile is withdrawn and as the labella come together, they trap an enclosed film of blood of about 10 microliters that are protected from drying (Foil,

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1987). Tabanids are able to be mechanical vectors of many pathogens (Foil, 1989) including *B. anthracis*. They can carry anthrax bacilli from anthrax-infected animals and disseminate spores by means of their wings and legs. Structurally, the tabanid is well adapted for collecting pathogens. Its stout legs end in three pads-like hairs that readily collect spores and disseminate them by contact. The well developed tabanid's wings can also be used for spore spreading. Tabanids have an intrinsic flight range of over 50 km (Hocking, 1953) and may play a role in the dissemination of spores and bacilli in a wide area, contributing to the epidemic of Anthrax.

Lack of rapid and efficient control measures, as well as irregular vaccination programs against the disease, could also contribute to occurrence of large outbreaks in the Chari-Baguirmi Province, sick animals are slaughtered and meat usually consumed with or without prior veterinary inspection, even in case of confirmed Anthrax cases. Part of the meat is eaten fried or dried for further consumption. The skin is removed for sale. Carcass is neither disposed nor burned as having fuel is often problematic in the area. This further can help to diffuse the disease agent by various flies.

The Direction de l'Élevage et des Ressources animales (DERA, 2000) have reported Anthrax in the following villages: Chilo, Balarye, Abardjl, Ousmanari, Ambague, Kiessa-Cherif and Kiessa-Hassan.

Both animals and humans have been seriously affected (WHO, 1998, N'Gamandje, 1984, Lamarque, 1990, Aba, 1997 and DERA, 1996, 1998). In 1998, the DERA reported cases of mortality among the population. This makes *B. anthracis* an important public health issue in a country where biting flies populations are varied and numerous.

Although, *B. anthracis* can be transmitted through biting flies, none bacillus has been so far isolated from them. This study aimed to determine the role of tabanids in the transmission of Anthrax in a region known as enzootic Anthrax zone. The second purpose of the present work was to examine the different isolates of *B. anthracis* collected from human, cattle and tabanid origin using various techniques. A biological and biochemical characterization of each isolates was carried out based on standard protocol. Susceptibility to antibiotics was also determined for each isolates.

## 2. Materials and Methods

### 2.1. Materials

Nine films of blood from suspected anthrax-infected cattle, 4 swabs from inflammatory fluid from 4 humans bitten by tabanids and 1499 tabanids collected from the infested area using fly nets were used for this study.

### 2.2. Methods

#### 2.2.1. Bacteriology

Biological characterization was done in triplicates using the following procedure :

Direct microscopic examination of the suspected materials (swabs, films and dissected tabanid tissues) was performed using the methods of Gram, Soltys (1960) and Morris (1955).

These materials were grown in nutrient broth, selective medium of Morris, aired solid nutrient agar and 5% Columbia sheep blood agar and incubated at 37°C, for 48 hours; stained by Gram as well as Soltys and examined by direct microscopy.

The pure isolates were inoculated subcutaneously with 0.5 ml, 1.0 and 0.25 ml into each laboratory animal, i.e. 8 guinea pigs, 6 rabbits and 10 mice, respectively, to determine the pathogenicity amongst other characteristics of the isolates.

#### 2.2.2. Biochemistry

Biochemistry characterization was done using the commercial biochemical reaction typing tools API 50 CH and API 20 £ containing together 49 substrates according to the manufacturer's protocols (API system S.A – Montialieu Vercieux – France).

#### 2.2.3. Sensitivity Test

Antibiotic susceptibility testing was carried out using the antibiogramme BIO-DISC (Biomerieux- France) according to the manufacture's instructions. The results were interpreted according to the international guidelines as described by Maho, 2006.

#### 2.2.4. Mobility Test

Semi-solid agar (nutrient broth with 0.3% agar) in culture tubes have been inoculated by the isolates. Inoculations were made by the stab method with a straight needle. Incubation was carried out at 37°C, for 6 days.

## 3. Results

About 89% of the 1499 tabanids examined were contaminated by anthrax-like organisms. *B. anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%) (Table 1).

Inoculation of the nutrient broth cultures by the humans inflammatory fluid, cattle blood and tissues from tabanids showed floccular growth on the surface of the cultures which sinks to the bottom within 24 hours of incubation. Isolates were non hemolytic on 5% Columbia sheep blood agar. Dull opaque, grayish-white colonies, with an irregular border was observed on aired solid nutrient agar media. Upon Gram-staining, long chains encapsulated, gram positive bacilli with rod shape were observed. These isolates were non motile and sensitive to penicilline (Table 2).

When the isolates were stabbed into Motility Test medium, growth occurs only along the line of inoculation.

The 30 laboratory animals inoculated subcutaneously with isolates died within 24-72 hours post-inoculation, i.e. guinea pigs (n = 10, 100% mortality), mice (n = 9, 90% mortality) and rabbits (n = 9, 90% mortality), indicating that isolates could be fully pathogenic to laboratory animals.

Autopsy carried out on guinea pigs showed that the tissues were swarming with gelatinous infiltration beneath the skin of the abdomens. The spleens were hypertrophied with dark uncoagulated blood.

Edematous areas on the livers, spleens, hearts and kidneys were detected. While staining these tissues,

regularly encapsulated bacilli were observed as single cells, in cluster or short chains. The spread of blood from guinea heart on solid agar medium showed a colony of *B. anthracis*-like within 24 hours of incubation at 37°C.

All isolates were sensitive to meticcillin, tetracycline, nitrofurantoin, sulphathiazol, piperacillin, benzathienepenicillin, chloramphenicol and oxytetracycline, and resistant to fusidic acid, polymyxin, sisomicine and

clindomycin. Intermediate reactions were found against netilmicin, streptomycin, rifampicin, pipemidic acid, cefalotin and sulfamids.

Biochemical characterization of the strains yielded similar results for all isolates with 48 out of 49 tests. Both human and cattle isolates showed negative reaction for Arbutine in contrast to those of tabanids (which tested positive) (Table 3).

**Table 1.** Distribution of spores and bacilli of *B. anthracis* in different tissues of dissected tabanids (-, neither spores nor bacilli)

Villages from which tabanids were collected	Total and Percent (%)	Number of tabanids examined	Number of tabanids showing spores on their wings and legs		Number of tabanids showing Bacilli in their mouthparts and midguts		Number of tabanids showing neither spores nor bacilli
			wings	Legs	mouthparts	Midguts	
			Chilo	824	351	206	
Balarye	205	-	49	112	34	10	
Abardjil	207	74	-	75	52	6	
Ousmanari	97	-	-	56	37	4	
Ambague	109	49	52	-	-	8	
Kiessa- Cherif	26	-	11	4	9	2	
Kiessa-Hassan	31	5	13	11	-	2	
Total	1499	479	331	282	245	162	
Percent (%)	100%	31.9	22.1	18.8	16.3	10.8	

**Table 2.** Biological properties of isolated *B. anthracis* from humans, cattle and tabanids (-, negative reaction; +, positive reaction)

Biological properties	Reactions		
	Humans	Cattle	Tabanids
Shape	rod	rod	rod
Capsulation	+	+	+
Gram staining	+	+	+
Mobility	-	-	-
Hemolysine	-	-	-
Pathogenicity	+	+	+
Growth on nutrient broth and aired solid nutrient agar medium	+	+	+
Sensitivity to penicilline	+	+	+

**Table 3.** Similarities and differences in Biochemical properties of isolated *B. anthracis* from humans, cattle and tabanids, by using different substrates

REACTIONS			
Substrate	Humans	Cattle	Tabanids
D-raffinose	-	-	-
Galactose	-	-	-
Salicine	-	-	-
D-glucose	+	+	+
Maltose	+	+	+
Ribose	+	+	+
Saccharose	+	+	+
Arbutine	-	-	+
Amidon	+	+	+
Glycogene	+	+	+

Only the main differences and similarities between human, bovine and tabanids are indicated: -, negative reaction; +, positive reaction.

#### 4. Discussion

During the past few years, *B. anthracis* has been one of the main bacterial zoonosis reported in Chad. High cattle density and abundant tabanids population present in N'Djamena rural area favour the occurrence of large outbreaks in the province.

The potential role of tabanids in the spread of *B. anthracis* to humans and domestic animals during an anthrax outbreak has been highlighted in a few studies (Lamarque, 1990; Sirol, 1971; Davies, 1985). However vegetative cells or spores have never been isolated from these vectors. Choquette (1983) and Davies (1983) reported that, anthrax spore may spread within a geographic region through insects feeding on infected animals.. Tabanids as well as *Stomoxys calcitrans*, *Musca domestica*, and *Calliphora erythrocephala* have been evaluated as transmitting agents (De Vos, 1998 and Dragon, 1999).

In this study *B. anthracis*-like strains were isolated from Tabanids and human bitten by tabanids. About 89% of the 1499 tabanids examined were contaminated by both spores and bacilli. *B. anthracis* spores were recovered from wings (31.9%) and legs (22.1%). Vegetative cells were recovered from mouthparts (18.8%) and midguts (16.3%). This result suggests that tabanids may play a role in the dissemination of anthrax spores and bacilli (either mechanically or through bite) in a wide area and contribute to the epidemic of anthrax within the Chari-Baguirmi Province of Chad. The infected areas can present a persistent public health risk to surrounding population. The different isolates collected throughout this work from humans, cattle and tabanids were identified as *B. anthracis* strains based on their biological and biochemical properties. They were distinguished from closely related species such as *Bacillus cereus*, *Bacillus pumilus* and

*Bacillus stearothermophilus*, as they are non motile and show positive reactions to glycogen and starch. They also differed from other non-pathogenic, aerobic spore-former species such as *Bacillus mesentericus*, *Bacillus mycoides*, *Bacillus brevis*, *Bacillus coagulans* and *Bacillus megaterium* which are mobile and haemolysin producer. Curiously, all *B. anthracis*-like isolates showed similar biochemical properties, except for Arbutine. 20-80% variability in Arbutine profile are reported for *B. anthracis* (Logan, 1984). Although isolates from tabanids tested positive to Arbutine, in contrast to those from humans and cattle, we believe that this discrepancy might be due to some artifacts. However, additional PCR confirmation and typing would be helpful to ascertain the species identification and to point out the route of transmission.

## 5. Conclusion

To diminish the risk associate with anthrax for humans, in N'Djamena rural area, we recommended wide spread animal vaccination (Marty, 2001).

Fly control should also be considered a part of an Anthrax control program, along with appropriate measures to promptly eliminate infected animals and carcasses. In addition, Health officials should use the set of antibiotics evaluated in this study, as recommended in other studies (Aubry, 1980, Frean, 2003 and Lamarque, 1990).

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