Detection of Antibodies Against Rabies Virus in Foxes Serum after Oral Vaccination Campaign in Buzău and Galați Counties

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Abstract

Rabies is one of the most feared zoonotic diseases worldwide, caused by a virus of the genus *Lyssavirus*, *Rhabdoviridae* family. In order to evaluate the efficacy of oral vaccination campaigns of foxes against rabies, two methods are agreed by WHO and OIE, namely: the quantification of rabies antibodies and the detection of a biomarker (tetracycline).

The aims of this study were consisting in the identification and quantification of rabies antibodies in foxes (*Vulpes vulpes*) after oral vaccination campaign in Buzau and Galati Counties.

After 2015 wildlife rabies vaccination campaign, 27 blood samples from hunted foxes were collected in Buzau (19 samples) and Galati (8 samples) Counties. According to manufacturer's recommendations, rabies seroconversion was quantified using PlateliaTM Rabies II Kit (Bio-Rad, France). In both counties were identified seropositive foxes following vaccination. Out of 19 samples from Buzau County, 3 samples overcome the requested seroconversion level (0.736 EU/ml, 0.933 EU/ml and 2.362 EU/ml), another 5 didn't reach the seroconversion level according to PLATELIATM RABIES II test and 11 samples had undetectable seroconversion (<0.125 EU/ml). Out of 8 samples from Galati County, 3 overcome the requested seroconversion level (2.963 EU/ml, 3.177 EU/ml and 3.456 EU/ml), 2 samples didn't reach it (0.372 EU/ml and 0.439 EU/ml) and 3 samples had undetectable seroconversion (<0.125 EU/ml). Only 22.22% of serum samples overcome the requested level of rabies antibodies, and 25.93% remained under the requested level, according to PLATELIATM RABIES II test. The results suggest that additional oral vaccination campaigns should be implemented in order to increase the proportion of foxes with optimal seroconversion level of rabies antibodies.

Keywords: foxes, oral vaccination, rabies

INTRODUCTION

Rabies is a viral zoonosis caused by a virus of the genus *Lyssavirus*, *Rhabdoviridae* family, which is responsible for a mortality rate of almost 100%, known as one of the most feared zoonotic diseases worldwide (Sudhi Ranjan Garg, 2014).

Although half of the world's population lives in endemic areas, more than 95% of the world's fatal cases of rabies occur in Africa and Asia, which are the continents with the highest risk of human mortality (OIE, 2015).

An effective rabies control strategy can be achieved through collaboration and communication between countries and by applying the same strategies, which might consist in health education campaign on rabies control, domestic and wild animals' rabies vaccination campaigns, and improvement of access to medical care (Singh *et al.*, 2011; OIE, 2015).

Starting July 3rd, 2004, a new European regulation has been taken regarding the international movements of domestic carnivores from rabies infected countries to rabies-free countries. In this respect, domestic carnivores are allowed to travel if their serum contains at least 0.5 IU/ml (International Units/ml) of rabies neutralizing antibodies. For that purpose, many rabies free countries have adopted a scheme requiring the rabies vaccination of pets associated with a serological test in order to prevent any introduction of rabies (Servat *et al.*, 2006; Regulation (EU) No 576/2013).

After January 1st, 2007, our country, as a member state of the European Union, has been obligated to adopt the strategy and concept of the oral vaccination of fox's in accordance with the EU legislation and with those recorded in the report of the Scientific Committee on Animal health and Animal welfare from October 23 of 2002. In this way, it was adopted the Government Decisionno.55/2008 for the approving of the Strategic Program regarding the surveillance, control and eradication of rabies in foxes in Romania for a period of 10 years. The objectives of this program are represented by an implementation of rabies control policy through fox vaccination and by a control of fox population and ultimately by the eradication of this disease (H.G. no. 55/2008).

The effectiveness of oral vaccination campaigns in foxes (*Vulpes vulpes*) is usually evaluated by two methods: (1) the quantification of rabies serum antibodies and (2) the vaccine biomarker (tetracycline) detection in teeth/bones (Wasniewski *et al.*, 2014a). Anti-rabies antibodies titration can be used to assess the effectiveness of the vaccination campaigns and in the determination of immune status in individual animals for regulation of international movement or trade (Lupulovic *et al.*, 2015; Wasniewski *et al.*, 2014b). The detection of a biomarker incorporated into the vaccine bait is considered

a useful tool used in conjunction with serological exam. However, it does not provide information about the post-vaccination immune response of the foxes and if is applied alone may lead to an overestimation oral vaccination efficacy. In several European countries, oral vaccination campaign led to the elimination of rabies among red foxes (*Vulpes vulpes*) (De Benedictis *et al.*, 2012).

The aims of this study were identification and quantification of rabies antibodies in foxes (*Vulpes vulpes*) after oral vaccination campaign in Buzau and Galati Counties.

MATERIALS AND METHODS

2.1 Samples

- 19 blood serum samples from foxes hunted in Buzau County
 - Basca Mare
 - Aldeni
 - Rusetu
 - Siriu
 - Amara
 - Luciu
 - Braesti
- 8 blood serum samples from foxes hunted in Galati County (fig.1)
 - Dedulesti
 - Calnau
 - Moieni
 - Boldu
 - Paltinis
 - Beceni
 - Piciorul Caprei

These fox blood samples collected with the purpose of antibodies detection were with a varying quality, based on time of collection post-mortem, storage temperature, the condition of the carcass before sampling and the environmental condition, like the season of oral vaccination campaign.

Fig.1. Romanian map and the geographical position of Galati and Buzau Counties.

2.2 ELISA – *Platelia™ Rabies II kit ad usum Veterinarium*

For the detection of rabies virus antigen glycoprotein antibodies from the fox serum samples, an indirect immune-enzymatic assay (*Platelia™ Rabies II kit ad usum Veterinarium*, Biorad, France) was used according to manufacturer's recommendations and instructions.

This test that may be performed for domestic carnivores and wildlife was developed by Bio-Rad (Marnes-La-Coquette, France) and ANSES Nancy Laboratory in 2007 and the specificity and sensitivity of this test were evaluated as being 98.6% and 88.8% respectively.

Comparing to other techniques, the aforementioned one has several advantages that are consisting in: the rapidity in obtaining the results (<3 hours), simplicity in comparison to virus neutralization, the safety regarding the manipulation (because doesn't involved handling live rabies virus) and the quantitative and qualitative results that can be obtained.

Briefly, the diluted serum samples, the positive and negative controls and the quantification standard were distributed into the microplates and then incubated at 37°C for one hour. To remove unbound antibodies and other proteins of the samples after incubation, 3 washings steps were performed. Next step was consisting in adding 100 µl conjugate- protein A labeled to peroxidase and purified bovine protein to each well, followed by a second incubation step at 37°C for one hour and 5 washings steps in order to remove unbound conjugate. The presence of the immune complexes

was highlighted by adding to each well a peroxidase substrate solution (which is a solution of citric acid and sodium acetate containing 0.015% H₂O₂ and 4% dimethylsulfoxide) and a chromogen (a tetramethylbenzidine solution 0.25%) followed by incubation at room temperature for 30 minutes and the addition of 100 µl solution of H₂SO₄ 1N to stop the enzymatic reaction. The microplates were read bichromatically at 450 and 620 nm.

Qualitative interpretation of results consist in comparison of samples OD with positive controls OD and classification of samples in 3 different categories, namely: seroconverted (+++), seroconverted and not seroconverted. For the quantitative determination of anti-rabies antibodies was made a standard curve by using standards and controls supplied on the kit, and the titres of serum samples were expressed in Equivalent units per ml (EU/ml) (Fig. 2). The results of our determinations were included in the next categories: high seroconversion level (>4 EU/ml), sufficient seroconversion level (0.5-4 EU/ml), insufficient seroconversion level (0.125-0.5 EU/ml) and undetectable seroconversion.

RESULTS AND DISCUSSION

3.1 Qualitative interpretation of results

All results were validated in accordance with the manufacturer's recommendations. In both counties, seropositive foxes following vaccination were identified. The results of qualitative determination of seroconversion for samples collected in Buzău County are shown in Tab. 1 and Fig. 3.

Fig. 2. Standard curve for the quantitative determination of anti-rabies antibodies in Equivalent units per ml obtained with standards and controls supplied by *Platelia™ Rabies II kit ad usum Veterinarium*, Biorad, France (edited with Rabies QT-ELISA BIORAD-Vers.200712.A.XLS)

For the category seroconverted (+++) regarding the 19 serum samples coming from Buzau County, no sample did framed it, while for the category seroconverted, 3 of them were positive.

The rest of the 16 samples were negative, being not seroconverted.

Tab.1. The Townships of Buzau County with positive results of the fox's serum samples

County	Township	No. of samples	Positive/negative	Criteria Result Validation
Buzau	Beceni	1	+	Seroconverted
	Moieni	1	-	Not seroconverted
	Basca Mare	2	+	Seroconverted
			-	Not seroconverted
	Calnau	1	-	Not seroconverted
	Aldeni	1	-	Not seroconverted
	Rusetu	2	-	Not seroconverted
			-	Not seroconverted
	Braesti	1	+	Seroconverted
	Boldu	1	-	Not seroconverted
	Amara	1	-	Not seroconverted
	Siriu	1	-	Not seroconverted
	Luciu	2	-	Not seroconverted
			-	Not seroconverted
	Piciorul Caprei	2	-	Not seroconverted
			-	Not seroconverted
	Dedulesti	2	-	Not seroconverted
			-	Not seroconverted
	Paltinis Casoca	1	-	Not seroconverted
Total		19	3/16	-

Fig. 3. Results of the tested fox's serum samples from Buzau County

The results of qualitative determination of seroconversion for samples collected in Buzau County are shown in Tab. 2 and Fig. 4.

For the category seroconverted (+++) regarding the 8 serum samples coming from Galati County, no sample did framed it. For the category seroconverted, 3 of them were positive, while the rest of the 5 samples were negative, being not seroconverted.

3.2 Quantitative interpretation of results

To determinate which is the quantity of anti-rabies antibodies in each sample, a comparison of the optical density of the samples to a standard curve was performed. The serum titre of all samples was obtained after a direct reading on the standard curve and was expressed as Equivalent

Units per millilitre (EU/ml), representing the quantitative determination.

Out of 19 samples from Buzau County, three samples overcome the requested seroconversion level (0.736 EU/ml-Beceni; 0.933 EU/ml-Basca Mare and 2.362 EU/ml-Braesti), another five didn't reach the seroconversion level according to PLATELIA™ RABIES II test (0.204 EU/ml-Moieni; 0.274 EU/ml-Luciu; 0.359 EU/ml-Boldu; 0.374 EU/ml-Paltinis, and 0.522 EU/ml- Piciorul Caprei) and 11 samples had undetectable seroconversion <0.125 EU/ml, namely: the serum samples coming from Basca Mare, Calnau, Aldeni, Amara, Siriu, Rusetu (x2), Luciu, Dedulesti (x2), and Piciorul Caprei.

Three serum samples from Galati County overcome the requested seroconversion level

Tab.2. Positive results of the fox's serum samples coming from Galati County

County	No. of samples	Positive/negative	Criteria Result Validation
Galati	8	-	Not seroconverted
		-	Not seroconverted
		-	Not seroconverted
		-	Not seroconverted
		+	Seroconverted
		-	Not seroconverted
		+	Seroconverted
		+	Seroconverted
Total		3/5	-

Fig.4. Results of the tested fox's serum samples from Galati County

(2.963 EU/ml, 3.177 EU/ml and 3.456 EU/ml), two samples didn't reach it (0.372 EU/ml and 0.439 EU/ml) and three samples had undetectable seroconversion (<0.125 EU/ml) (fig.5).

Low level of seroconversion could be registered also in recently vaccinated animals, and undetectable seroconversion in recently vaccinated and unvaccinated animals, therefore this test should be carried out only on samples taken at least 30 days after vaccination.

For the serum samples coming from Buzau County, in 8 out of 19 were identified anti-rabies antibodies, but in only 3 of them overcome the requested seroconversion level. Even if the anti-rabies antibodies were found also in the others 5 serum samples, the antibodies level is insufficient for potency and doesn't provide a sufficient immunity to the animal. Also, the same situation was in Galati County, where even in 5 out of 9 samples were found anti-rabies antibodies, only

Tab. 3. Optical densities values of 27 fox's serum samples from Buzau and Galati Counties (data processed with Rabies QT-ELISA BIORAD-Vers.200712.A.XLS)

Controls and Standards		Sample ID (type sample IDs in grey fields)				
OD Values		OD Values (type OD values in yellow fields)				
1	2	3	4	5	6	7
A R3	S4 = 1 UE/ml	1.Beceni	9. Boldu	17.Dedulesti	Galati	
0,018	1,894	1,388	0,727	0,011	0,147	
B R3	S4 = 1 UE/ml	2.Moieni	10. Amara	18.Dedulesti	Galati	
0,013	1,873	0,459	0,032	0,046	2,867	
C R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	3.Basca Mare	11.Siriu	19.Paltinis	Galati	
1,2	1,029	1,758	0,020	0,749	2,940	
D R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	4. Basca Mare	12. Rusetu	Galati		
1,19	0,851	0,012	0,117	0,746		
E S6 = 4 UE/ml	S2 = 0.25 UE/ml	5. Calnau	13.Luciu	Galati		
3,11	0,576	0,041	0,598	0,097		
F S6 = 4 UE/ml	S2 = 0.25 UE/ml	6. Aldeni	14. Luciu	Galati		
3,054	0,549	0,040	0,039	0,032		
G S5 = 2 UE/ml	S1 = 0.125 UE/ml	7.Rusetu	Piciorul Cap	Galati		
2,816	0,27	0,101	0,982	0,848		
H S5 = 2 UE/ml	S1 = 0.125 UE/ml	8.Braesti	Piciorul Cap	Galati		
2,503	0,289	2,654	0,057	2,811		

Tab. 4. Antibody titer of 27 fox's serum samples from Buzau and Galati Counties (data processed with Rabies QT-ELISA BIORAD-Vers.200712.A.XLS)

Controls and Standards		Sample ID				
Titer (EU/ml)		Antibody Titer (EU/ml)				
1	2	3	4	5	6	7
A R3	S4 = 1 UE/ml	1.Beceni	9. Boldu	17.Dedulesti	Galati	
<0.125 EU/ml		0,736	0,359	<0.125 EU/ml	<0.125 EU/ml	
B R3	S4 = 1 UE/ml	2.Moieni	10. Amara	18.Dedulesti	Galati	
<0.125 EU/ml		0,204	<0.125 EU/ml	<0.125 EU/ml	3,177	
C R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	3.Basca Mare	11.Siriu	19.Paltinis	Galati	
0,638		0,933	<0.125 EU/ml	0,374	3,456	
D R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	4. Basca Mare	12. Rusetu	Galati		
0,632		<0.125 EU/ml	<0.125 EU/ml	0,372		
E S6 = 4 UE/ml	S2 = 0.25 UE/ml	5. Calnau	13.Luciu	Galati		
		<0.125 EU/ml	0,274	<0.125 EU/ml		
F S6 = 4 UE/ml	S2 = 0.25 UE/ml	6. Aldeni	14. Luciu	Galati		
		<0.125 EU/ml	<0.125 EU/ml	<0.125 EU/ml		
G S5 = 2 UE/ml	S1 = 0.125 UE/ml	7.Rusetu	Piciorul Cap	Galati		
		<0.125 EU/ml	0,522	0,439		
H S5 = 2 UE/ml	S1 = 0.125 UE/ml	8.Braesti	Piciorul Cap	Galati		
		2,362	<0.125 EU/ml	2,963

Fig. 5. The quantity of anti-rabies antibodies in 27 fox's serum samples from Buzău and Galați Counties determined by comparing the optical density of the sample to a standard curve. >4 EU/ml: high seroconversion level; 0.5 - 4 EU/ml: sufficient seroconversion level; 0.125 - 0.5 EU/ml: insufficient seroconversion level; <0.125 EU/ml: undetectable seroconversion. According to PLATELIA™ RABIES II test specifications, insufficient or undetectable seroconversion can possibly be reported in recently vaccinated animals, due to delayed immune response.

3 of them overcome the requested seroconversion level, the others 2 having an insufficient antibodies level.

CONCLUSION

Only 22.22% (6/27) of serum samples overcome the requested level of rabies antibodies, while 25.93% (7/27) remained under the requested level. The results suggest that additional oral vaccination campaigns should be implemented in order to increase the proportion of foxes with optimal seroconversion level of rabies antibodies. Therefore, counting the history of the Romanian fox vaccination campaign and the other states experience, it became obviously the requirement for an extended vaccination in a continuously manner over few years, in order to receive a good coverage in population. Oral vaccination in red foxes (*Vulpes vulpes*) should be regarded as an important support to traditional prevention and control measures in rabies.

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