EARLY EXCRETION OF RABIES VIRUS IN SALIVA OF FOXES AND DOGS.

J. Barrat¹, M. Aubert¹ and J. Blancou²

1 INTRODUCTION.

The classical vehicle of rabies virus during the transmission of the disease is saliva. Several techniques give estimations of the shedding of rabies virus in saliva.

When examining dead animals data are fixed on the moment of death, the examination of salivary glands shows then the presence of the virus or of its antigens in the gland tissue without information of what is in saliva itself, the examination of saliva may also be difficult because of a small amount of saliva or heavy bacterial contamination.

Sampling saliva on alive animals on the moment of euthanasia gives the same kind of data as the ones collected on dead animals, but it is then possible to collect better specimens of saliva and larger volumes.

The regular examination of experimentally inoculated or suspect restrained animals gives more interesting information on the kinetics of excretion but needs an assessed sampling system.

2 PRESENCE OF VIRUS IN SALIVARY GLANDS OF RABID ANIMALS.

2.1 Presence of virus in salivary glands of naturally infected animals.

In canine rabies area, Vaughn et al. (1965) isolated rabies virus in 28 out of 45 dogs (62%) with a mean titre of $10^{4.5}$ mouse intracerebral lethal dose for 50% animals (MICLD₅₀) per 0.03g of tissue.

In arctic and sylvatic rabies area, the results of salivary glands examinations are shown in Table 1.

Table 1 : isolation of rabies virus in salivary glands of naturally infected animals in arctic and sylvatic rabies area.

<table>
<thead>
<tr>
<th>species</th>
<th>positive / examined</th>
<th>mean titre*</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>red fox</td>
<td>22/101</td>
<td>n.d.</td>
<td>Selimov et al., 1980</td>
</tr>
<tr>
<td>arctic fox</td>
<td>23/198</td>
<td>4.5</td>
<td>Vaughn et al., 1965</td>
</tr>
</tbody>
</table>

* in log(MICLD₅₀ / 0.03g tissue)

In red fox sylvatic rabies area, red foxes, badgers, roe deers and stone martens were controlled for the presence of rabies virus in salivary glands (Table 2). These species correspond to the most frequent wildlife victims of sylvatic rabies and all of them except stone martens showed a high percentage of presence of rabies virus in salivary glands in these natural conditions. The comparison of the titres and percentages obtained in Switzerland (Wandeler et al., 1974) and in France 10 years later (Barrat, unpublished data) showed no difference neither in these wild species nor in domestic dogs. This absence or non detected evolution of the virus was experimentally confirmed on foxes (Aubert et al.; 1991) : two isolates of fox virus obtained on naturally rabid red foxes in 1976 and in 1986 in France induced rabies within the same mean delay between inoculation and death but the older strain did it with a larger dispersion of delays. Monoclonal antibodies showed a difference in epitopes of the site 2 of the nucleocapsid of the two strains.

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### Table 2: Isolation of Rabies Virus in Salivary Glands of Naturally Infected Animals in Sylvatic Rabies Area in Europe.

<table>
<thead>
<tr>
<th>Species</th>
<th>Positive / Examined</th>
<th>Mean Titre*</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red fox</td>
<td>18/21 (87.5%)</td>
<td>N.D.</td>
<td>Matouch, 1978</td>
</tr>
<tr>
<td>Dog</td>
<td>759/816 (93%)</td>
<td>3.4</td>
<td>Wandeler et al., 1974</td>
</tr>
<tr>
<td></td>
<td>17/20 (85%)</td>
<td>2.14</td>
<td>Original data, 1985</td>
</tr>
<tr>
<td>Roe deer</td>
<td>7/9 (78%)</td>
<td>1.4</td>
<td>Original data, 1985</td>
</tr>
<tr>
<td>Badger</td>
<td>68/82 (83%)</td>
<td>3.5</td>
<td>Wandeler et al., 1974</td>
</tr>
<tr>
<td></td>
<td>23/24 (96%)</td>
<td>3.4</td>
<td>Original data, 1985</td>
</tr>
<tr>
<td>Stone marten</td>
<td>18/36 (50%)</td>
<td>1.5</td>
<td>Wandeler et al., 1974</td>
</tr>
<tr>
<td></td>
<td>15/25 (60%)</td>
<td>1.29</td>
<td>Original data, 1985</td>
</tr>
</tbody>
</table>

* in log(MICLD50 / 0.03g tissue)

#### 2.2 Presence of Virus in Salivary Glands in Experimental Infections.

The inoculation of high doses of a red fox strain to red foxes does not show any correlation between the inoculated dose and the infection of salivary glands as shown in Table 3 (Blancou et al., 1979).

### Table 3: Isolation of Rabies Virus in Saliva and Salivary Glands after Experimental Infection of Red Foxes with a Fox Strain.

<table>
<thead>
<tr>
<th>Inoculated Dose of Virus</th>
<th>Virus in Saliva</th>
<th>Virus in Salivary Glands</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5.15 MIC LD50</td>
<td>3/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3.45 MIC LD50</td>
<td>3/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3.17 MIC LD50</td>
<td>3/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^3 MIC LD50</td>
<td>3/6</td>
<td>5/6</td>
</tr>
<tr>
<td>10^2.85 MIC LD50</td>
<td>2/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^2.72 MIC LD50</td>
<td>4/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^2.15 MIC LD50</td>
<td>3/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

#### 2.3 Presence of Virus in Salivary Glands of Dogs under Experimental Infections.

The same experiment conducted in dogs with a street strain isolated in a dog Morocco in 1985 gave similar results (Blancou et al., 1990):

### Table 4: Isolation of Rabies Virus in Salivary Glands after Experimental Infection of Dogs with a Dog Strain.

<table>
<thead>
<tr>
<th>Dose of Virus</th>
<th>Virus in Salivary Glands</th>
<th>Titre of Virus in Salivary Glands (MIC LD50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4.8 MIC LD50</td>
<td>2/3</td>
<td>10^0, 10^2</td>
</tr>
<tr>
<td>10^2.8 MIC LD50</td>
<td>2/3</td>
<td>10^-2, 10^2</td>
</tr>
<tr>
<td>10^0.8 MIC LD50</td>
<td>3/3</td>
<td>10^0, 10^-2, 10^-4</td>
</tr>
<tr>
<td>10^-2.2 MIC LD50</td>
<td>1/1</td>
<td>10^-1</td>
</tr>
</tbody>
</table>

#### 2.4 Conclusion.

The presence of virus in salivary glands depends on several parameters such as the host species, the strain of virus, the length of incubation and the inoculated dose. This presence of virus determines the co-adaptation in the host-virus system.

But the sole examination of salivary glands does not bring any information on the kinetics of the excretion: when did it begin?, is it constant?
3 DETECTION OF RABIES VIRUS IN SALIVA OF FOXES UNDER EXPERIMENTAL CONDITIONS.

3.1 Material and method.

3.1.1 Animals.

3.1.1.1 Red foxes.

Wild red foxes were captured in May-June (i.e. at 2 to 4 month of age) in rabies free areas in France. The animals were first put in quarantine till autumn and then experimented. At the beginning of any experiment, the absence of rabies neutralising antibody is controlled on every fox involved in the experiment.

3.1.1.2 Mice.

Four to six week old females OF1 specific pathogen free mice were purchased from IFFA Credo (69210 L'Arbresle, France) for back titration of the challenge virus. The presence of virus in saliva was assessed by intra-cranial inoculation to 2 days old suckling OF1 mice.

3.1.2 Virus strains.

3.1.2.1 GS3-1 fox strain.

GS3 batch is an homogenate of sub-maxillary salivary glands of naturally infected red foxes submitted for rabies diagnosis in 1976. The salivary glands have been homogenised at a 1:5 (w/v) dilution. The supernatant is then aliquoted in 0.75 ml glass ampoules that are immersed in liquid nitrogen. Two days later, one ampoule is thawed and titrated intracerebrally in mice. This batch has been passaged on one single fox whose sub-maxillary salivary glands have been used to prepare the GS3-1 batch according to the same procedure.

3.1.2.2 GS7-1 fox strain.

Sub-maxillary glands of naturally infected foxes in 1986 have been used to prepare the GS7 batch. This batch was passaged on one single fox to prepare the GS7-1 batch. GS7-1 was passaged in another fox to prepare GS7-1-1 batch.

3.1.2.3 Ma85 dog strain.

This batch has been prepared identically from sub-maxillary salivary glands of naturally infected dogs in Morocco in 1985.

3.1.3 Sampling tools.

Saliva specimens were collected using a "salivette" kit (Sarstedt, Molsheim, ref 51.1534). Blood specimens are collected at the jugular vein on vigil foxes manually restrained. Once the clot is formed, tubes are centrifuged and the serum is kept frozen at −30°C till the analysis.
3.1.4 Diagnosis tests.

When animals died, submaxillary salivary glands and brain were examined by FAT (Dean and Abelsett in Laboratory techniques in rabies, 1973) and cell culture test (Barrat et al., 1988). Neuroblastoma cells and suckling mice were used to detect rabies virus in saliva.

3.2 Results.

3.2.1 Assessment of the saliva collection system.

The ideal saliva sampling system must:

- be sterile to avoid contamination of the sample for inoculation tests
- allow a precise determination of the weight of saliva that has been collected to be able to determine the titre of the saliva
- protect rabies virus from inactivating factors like heat, light, dryness
- allow a recovery of diluted saliva as complete as possible.

A saliva sampling system (salivette, ref 51-1534, Sarstedt) which is classically used for man was adapted to fox for this purpose.

In order to estimate the decrease in the virus titre of the saliva due to the sampling system, the following preliminary experiment was conducted.

Saliva has been collected in healthy foxes that received a subcutaneous injection of pilocarpin (2 mg per animal).

A serial dilution of a virus suspension was prepared in cell culture medium (DMEM) for a classical LD_{50} determination in mice. Fifty µl of each dilution were then mixed with the same volume of pure saliva and 50µl of these spiked saliva dilutions were distributed on the cotton swab of a salivette. One minute later (i.e. the average time between collection of saliva on the fox and dilution of this saliva with DMEM in the experimental station) 200µl of DMEM were added (1:5 final dilution).

The salivettes were then centrifuged (3000 g at +4°C) to recover the spiked saliva. A new IC titration was then performed in mice with the recovered spiked saliva.

Four assays were performed. Results are given in Table 5.

**Table 5 : Titre reduction observed with the Salivette system.**

<table>
<thead>
<tr>
<th>Test</th>
<th>1</th>
<th>VS31</th>
<th>VS32</th>
<th>VS33</th>
<th>VS34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial mouse titre in log(MICLD_{50}/ml)</td>
<td>5.89</td>
<td>6.05</td>
<td>6.05</td>
<td>6.05</td>
<td>6.05</td>
</tr>
<tr>
<td>Final suckling mice titre log(MICLD_{50}/ml)</td>
<td>5.48</td>
<td>5.04</td>
<td>5.41</td>
<td>4.76</td>
<td>5.5</td>
</tr>
<tr>
<td>Decrease in titre in log</td>
<td>0.41</td>
<td>1.01</td>
<td>0.64</td>
<td>1.29</td>
<td>0.55</td>
</tr>
</tbody>
</table>

The observed decrease in titre ranged between 0.41 and 1.29, the average decrease is 0.78 log (i.e. the titre is divided by 2.5 to 19.5 with a mean of 6).

3.2.2 Excretion of rabies virus in foxes.

3.2.2.1 Experiment one.

The experiment (Blancou et al., 1990) involved 40 foxes in two groups. In each group, 10 pairs of animals were experimented, each pair was composed of an inoculated animal and a contact one. These 7 to 11 month old foxes were paired 3 to 6 weeks before the beginning of the experiment to avoid any "behavioural" fight. In every cage, one fox received 1 fox IM LD_{100} (i.e. nearly 10^{1.5} mouse IC LD_{50}) in
The back titration on mice of the virus suspension showed that animals of the first group received $10^{1.9}$ mouse IC LD$_{50}$ of GS3-1 strain and $10^{1.5}$ mouse IC LD$_{50}$ of GS7-1. The second group of foxes was inoculated with $10^{1.9}$ mouse IC LD$_{50}$ of GS3-1 strain and $10^{1.8}$ mouse IC LD$_{50}$ of GS7-1. These titres are not different from the expected theoretical dose of $10^{1.5}$ mouse IC LD$_{50}$.

Saliva specimens were collected weekly during the first step of the experiment and twice a week during the second step.

No significant difference between strains GS7-1 and GS3-1 was noted regarding the excretion pattern in saliva and the titre in salivary glands (Aubert M. et al., 1991), that is why data are grouped.

In both inoculated and contact groups, thirty one foxes excreted the virus in saliva. The earliest detection occurred 14 days after inoculation in 2 inoculated animals and in one contact fox. Once excretion had begun, it was continuous for 17 foxes and intermittent for the 14 others.

The comparison with clinical data showed that excretion began 0 to 29 days before the clinical phase, i.e. 1 to 33 days before death (Figure 1).

**Figure 1 : Excretion pattern observed in 40 experimental foxes.**

<table>
<thead>
<tr>
<th>Days before symptoms</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>inoculating animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>contact animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 = absence of isolation of rabies virus in saliva
+
= isolation of rabies virus in saliva
grey squares = death of the animal
The highest percentage of foxes excreting rabies virus was observed at the onset of symptoms (Figure 2).

Figure 2: Percentage of foxes shedding rabies virus before and after the onset of symptoms.

![Figure 2: Percentage of foxes shedding rabies virus before and after the onset of symptoms.](image)

After the death of the animals, rabies diagnosis was performed, all 40 animals have been found positive for rabies. Rabies virus was isolated from sub-maxillary salivary glands of 34 animals. Two of the six animals whose salivary gland were negative excreted virus in saliva. These data are shown in Table 6

Table 6: Isolation of rabies virus in saliva on alive animal and in salivary glands after death.

<table>
<thead>
<tr>
<th>Isolation in saliva on alive animals</th>
<th>Isolation in salivary glands after death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative *</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
</tr>
<tr>
<td>Negative *</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
</tr>
</tbody>
</table>

* the dilution and homogenisation process used for the salivary glands gives a negative result when titre is under $10^{2.2}$ mouse IC LD$_{50}$ per gram of tissue

3.2.2.2 Experiment two.

In that experiment, one single fox was inoculated intramuscularly with 200 fox IM LD$_{100}$ (i.e. $10^{3.8}$ mouse IC LD$_{50}$) of GS7-1-1 strain. Saliva samples were taken daily between inoculation and death (20 days later).

Excretion of virus in saliva began 7 days after inoculation, i.e. 13 days before the onset of clinical signs. The excretion was detected every day up to the death.

When the fox died, brain and salivary glands were controlled positive for rabies.

4 Excretion of Rabies Virus in Dogs.

Five laboratory beagles have been inoculated with an homologous street strain, Ma85 batch. Each dog was inoculated in the temporal muscle with 1 ml of a Ma85 virus suspension containing $10^{3.5}$ mouse IC LD$_{50}$ (i.e. roughly 1 dog IM LD$_{80}$).

Specimens of saliva have been collected before and during the clinical phase and treated like the foxes ones. None of them allowed the isolation of rabies virus.

Four out of these five dogs died of rabies. The laboratory diagnosis was performed on these animals. The four dogs that died of rabies were positive in brain and only two of them were positive in salivary glands.
5 CONCLUSION.

The main conclusion of these experiments is that the shedding of rabies virus is hard to demonstrate on live animals. The follow-up of rabies virus excretion in saliva may be influenced by many factors related to:

- the technique used to detect the virus, saliva sampling and virus growth
- the ill animal, is it a natural case or an experimental one? If so, the different factors that may influence the shedding of rabies virus should be considered such as the strain that is studied, the route of inoculation and the dose, the frequency of saliva sampling

In the homologous system (red fox strain in red fox), the inoculated dose of red fox virus is related to the mortality delay in red fox. It has been shown here that foxes inoculated with a low dose of virus (1 IM LD₁₀₀) may excrete rabies virus in saliva very early in the course of the disease. This early excretion is also not continuous, at least with the detection tools used in this experiment.

The human and animal health and epidemiological implications of this very early excretion of rabies virus in saliva cannot be ignored.

REFERENCES


