Archiv für die gesamte Virusforschung 44, 361—369 (1974)

Herpesvirus Strigis, a New Avian Herpesvirus

II. Biochemical and Biophysical Properties

By

M. Sibalin, F. Gerstl, L. Pichler, and F. Bürki

Institute of Virology,
Veterinary University and Federal State Institute for Vaccine Production,
Vienna, Austria

With 8 Figures

Received February 2, 1974

Summary

A virus (HSIS) originating from dead owls was successfully cultivated in chicken embryo fibroblasts. Its replication was inhibited by IUDR. Tissue cultured virus proved sensitive to ether, chloroform, 0.5 per cent trypsin, and to pH levels of 4.0 or lower. Infectivity was rapidly destroyed at 56° C. Negatively stained naked virions of 100 nm average diameter were seen, and enveloped virions with 160—250 nm size. The capsid was built up of hollow cyclindrical capsomeres, arranged in equilateral triangles, carrying 5 capsomeres along each edge. Cubical symmetry and icosahedron structure yielded a total number of 162 capsomeres. All these biochemical and biophysical data lead to classification of HSIS virus into the genus herpesvirus. Biological properties described in a foregoing paper sustained such grouping, and indicated that the agent was a new avian herpesvirus for which the name *herpesvirus strigis* was proposed.

1. Introduction

A disease syndrom occurring in naturally infected owls and designated as *hepato-splenitis infectiosa strigum* (9) could be experimentally reproduced by a viral agent. In a foregoing paper (7) we classified this virus on behalf of its biological properties as *herpesvirus strigis*, a new avian herpesvirus. This paper describes its physicochemical properties.

2. Materials and Methods

2.1. Viruses

Herpesvirus strigis (HSIS), strain x/42/63, originally isolated from an Eagle Owl on CAM (9), was grown in chicken embryo fibroblasts and used in its 4th to 17th tissue culture passages.

Infectious laryngotracheitis virus (ILT), strain N 5256, was used in its 8th to 10th CAM passages.

Infectious bronchitis virus (IB), strain Beaudette, was used after an unknown number of egg passages in its 4th to 9th chicken fibroblast tissue culture passages.

Infectious bovine rhinotracheitis virus (IBR) strain LA, was used in its 13th passage on primary calf kidney monolayers.

Bovine enterovirus (ECBO), strain 51/60 (6), was propagated likewise and tested in its 17th passage.

Bovine rhinovirus, strain Sd-1 (4), was cultured in a roller drum at 33° C on primary calf kidney monolayers and was used in its 7th passage.

2.2. Tissue Culture and Chicken Egg Assay

Preparation and handling of tissue cultures and embryonated eggs has been described in the first paper (7). For all experiments reported in this paper serum-free tissue culture harvests were used. Taking into account the poor viability of herpesviruses at freezing temperatures in the absence of protective colloids, such harvests were stored at +4° C for short periods of time (19).

All titers recorded are expressed as TCD_{50} or ID_{50}/0.1 ml, computed according to REED and MUENCH (16).

2.3. Biochemical Tests

The inhibiting effect of IUDR on virus replication was measured in culture flasks (growth area 50 cm^2) by incorporating 25 µg/ml of 5-iodo-2-deoxyuridine (Sigma) into maintenance medium. Control flasks received normal maintenance medium. After a preincubation at 37° C for 3 hours, viruses were added in a multiplicity of approximately 1. One ml samples were withdrawn from each flask after 24, 48, 72 and 96 hours p.i. and immediately titrated for virus content.

Ether-resistance was determined according to ANDREWES and HORSTMAN (1), chloroform-resistance according to BÖGEL and MAYR (5).

Sensitivity to trypsin was tested according to DINTER (13), using crystallized, lyophilized sterile trypsin and 3× crystallized, lyophilized soybean trypsin inhibitor (Worthington Biochem. Corp. Freehold, N.Y., U.S.A.).

Sensitivity towards different low pH levels was determined as described by BÜRKI and PICHLER (8).

Temperature sensitivity was determined by keeping small aliquots of a given virus batch at —20° C for 48 hours, at +4° C for 48 hours, in a waterbath at 37° C for 24 hours, or for 15, 30, and 60 minutes respectively at 56° C. All samples were then kept at +4° C up to the time of titration for residual virus content. Such determinations were done concomitantly in a single batch of cell cultures.

2.4. Electron Microscopical Examination

Chicken embryo fibroblast flask cultures showing a CPE of 70 per cent (7) were decanted and the cell sheets collected with a rubber policeman in a small quantity of MILLONIG buffer (15). The cells were pelleted at 2500*g* for 5 minutes, washed twice with the same buffer and examined immediately.

After decanting the buffer, 4—6 drops of a 5 per cent ammoniumacetate solution containing 0.01 per cent of a 16 per cent bovine albumin solution were thouroughly mixed with sedimented cells (23). Two drops of this suspension were then mixed with 4 drops of a 2 per cent sodium PTA solution of pH 6.5 to 7.0. This mixture was transferred to Formvar grids previously fortified by a carbon layer. Photographs were made on Gevaert Scientia 23D50 plates in a Siemens Elmiskop I using 80KV and double condenser illumination.

3. Results

3.1. Nucleic Acid Type

As evident from Figure 8, the titer of HSIS virus in normal culture medium increased steadily over an observation period of 96 hours. In the presence of IUDR, however, no HSIS could be detected, except a low titer of probably residual input

virus in the sample taken at 24 hours p.i. This behaviour indicated that the essential nucleic acid of HSIS is DNA. On the other hand, IUDR failed to have any inhibiting effect on the replication of the RNA containing IB virus, a known coronavirus (2); the decrease in titer of IB virus observed after 72 hours p.i. might be due to thermal inactivation.

3.2. Resistance against Chemical and Physical Agents

According to Table 1, both herpesviruses tested, i.e. HSIS and IBR, got completely inactivated by ether as well as chloroform indicating that their virions were sensitive to lipid solvents. The ECBO virus, however, remained unaffected by both lipid solvents as an enterovirus should according to definition (2).

Table 1. *Results of Ether- and Chloroform-Resistance Tests*

	Virus content per 0.1 ml				
Viruses	Controls	Ether	Difference	Chloroform	Difference
HSIS	2.00[a]	<0.50	>1.50	<0.50	>1.50
IBR	6.00	<0.50	>5.50	<0.50	>5.50
ECBO	6.25	6.00	0.25	5.25	1.00

[a] $\log_{10}$ $TCID_{50}$.

Purified trypsin in 0.5 per cent concentration had a strongly inactivating effect on HSIS virus, as evident from Table 2. Likewise, IBR virus, a bovine herpesvirus determined earlier as sensitive to trypsin treatment (8) and retested for control purposes alongside to HSIS virus, was confirmed as being sensitive to trypsin. ECBO virus, known from previous experiments as resistant to trypsin (8), resisted to trypsin treatment as expected.

Table 2. *Results of Trypsin Sensitivity Tests*

	Virus content per 0.1 ml		
Viruses	PBS 60 min, 37° C	Trypsin 60 min, 37° C	Difference
HSIS	2.50[a]	<0.50	>2.00
IBR	6.75	1.00	5.75
ECBO	6.75	6.25	0.50

[a] $\log_{10}$ $TCID_{50}$.

Table 3 shows, that lowering the pH to 4.0 sufficed to abolish any infectivity of HSIS virus. The pH-lability of IBR virus (GRIFFIN *et al.*, cit. 2) or of a bovine rhinovirus (8), tested as controls was confirmed. ECBO virus, however, survived at even pH 2.2, thus meeting the property of a pH-resistant control virus (8).

As evident from Table 4, HSIS virus for short periods of time was stable at —20°, +4° or 37° C. It proved, however, very sensitive to heating at 56° C. IBR tested alongside displayed a similar temperature sensitivity fulfilling thereby another criterion of the herpesvirus group (2, 19). ECBO virus, on the other hand, partially withstood heating to 56° C even during prolonged incubation, this in accordance with previous reports (MOLL and FINLAYSON, cit. 2).

Table 3. *Results of Sensitivity Tests towards Low pH Obtained after 60 Minutes of Incubation at 37° C*

	Virus content per 0.1 ml			
Viruses	pH 7.0	pH 4.0	pH 3.0	pH 2.2
HSIS	3.75[a]	<0.50	<0.50	<0.50
IBR	7.25	<0.50	<0.50	<0.50
ECBO	6.75	7.25	6.50	6.75
Bov. Rhino	3.75	<0.50	<0.50	<0.50

[a] $\log_{10}$ $TCID_{50}$.

Table 4. *Results of Temperature Resistance Tests*

	Virus content per 0.1 ml					
Viruses	—20° C 48 hrs	+4° C 48 hrs	37° C 24 hrs	56° C 15 min	56° C 30 min	56° C 60 min
HSIS	3.00[a]	2.75	2.75	<0.50	<0.50	<0.50
IBR	n.t.[b]	5.75	4.75	<0.50	<0.50	<0.50
ECBO	6.50	6.00	6.00	2.00	1.50	0.75

[a] $\log_{10}$ $TCID_{50}$.
[b] Not tested.

3.3. Electron Microscopy

Using negative staining technique, about 60 per cent naked and 40 per cent enveloped HSIS virus particles were found. Their average size, based on a great number of determinations, measured 100 nm when naked, and 160—200 nm (occasionally up to 250 nm) when enveloped. Some capsids as well as most of their PTA-penetrated cores showed a hexagonal outline (Figs. 1, 4). The inner diameter of cores measured 75 nm on the average.

Within some virions core structures could be recognized. In some instances such structures contained a central ring approximating 35 nm in diameter and penetrated by PTA. (Fig. 1, upper left corner; Fig. 4.)

The capsomeres had the shape of hollow cylinders (Figs. 6 and 7) measuring 10 nm in length and 10 nm in width, with a central hole approximating 2.5 nm (Fig. 6). Capsomeres were arranged in equilateral triangles with 5 capsomeres along one edge (Fig. 7, arrows).

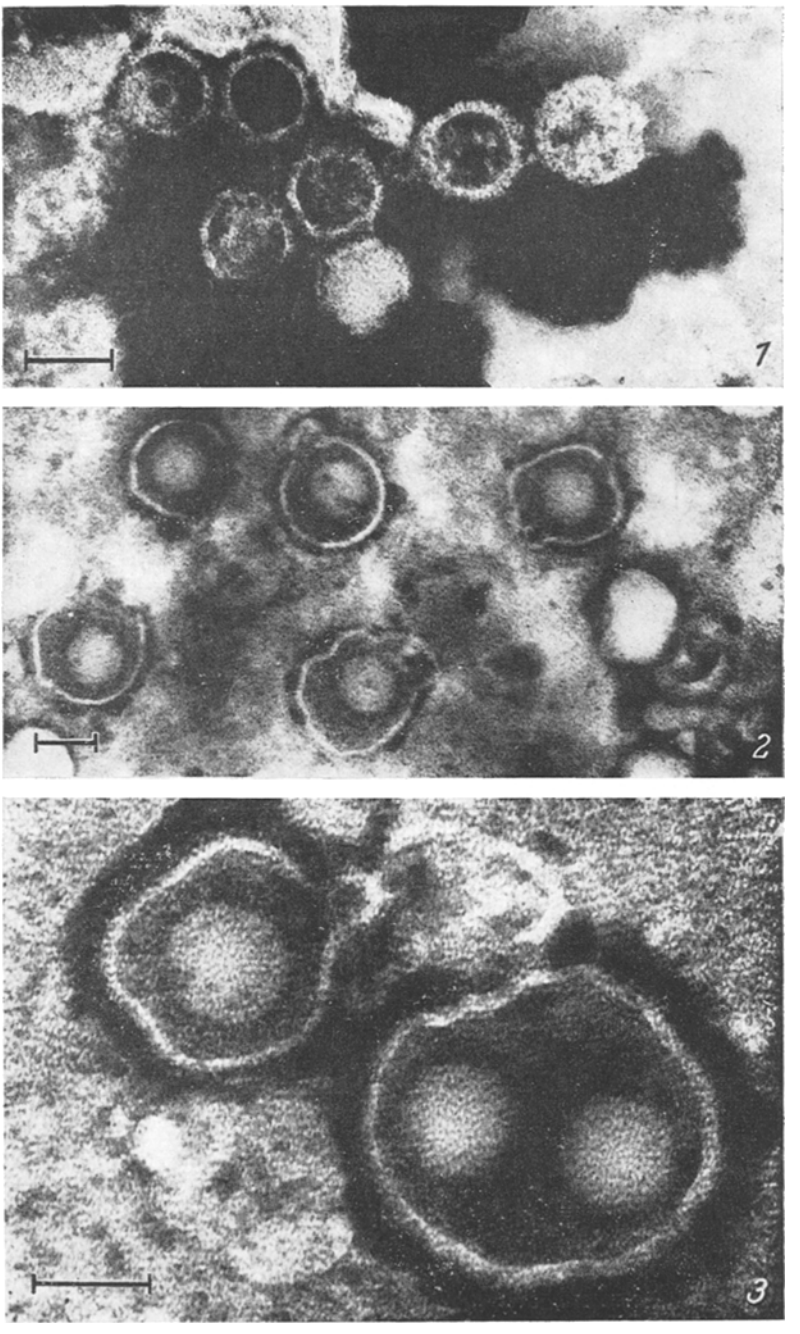

Plate 1. PTA-negatively stained electron-micrographs of herpesvirus strigis virions. Bars = 100 nm

Fig. 1. Naked virus particles, showing different contrasting effect. Several capsids have an hexagonal outline, some have an "empty", others a "full" appearance

Figs. 2, 3. Enveloped virus particles. In Figure 3 two capsids are enclosed in a common envelope

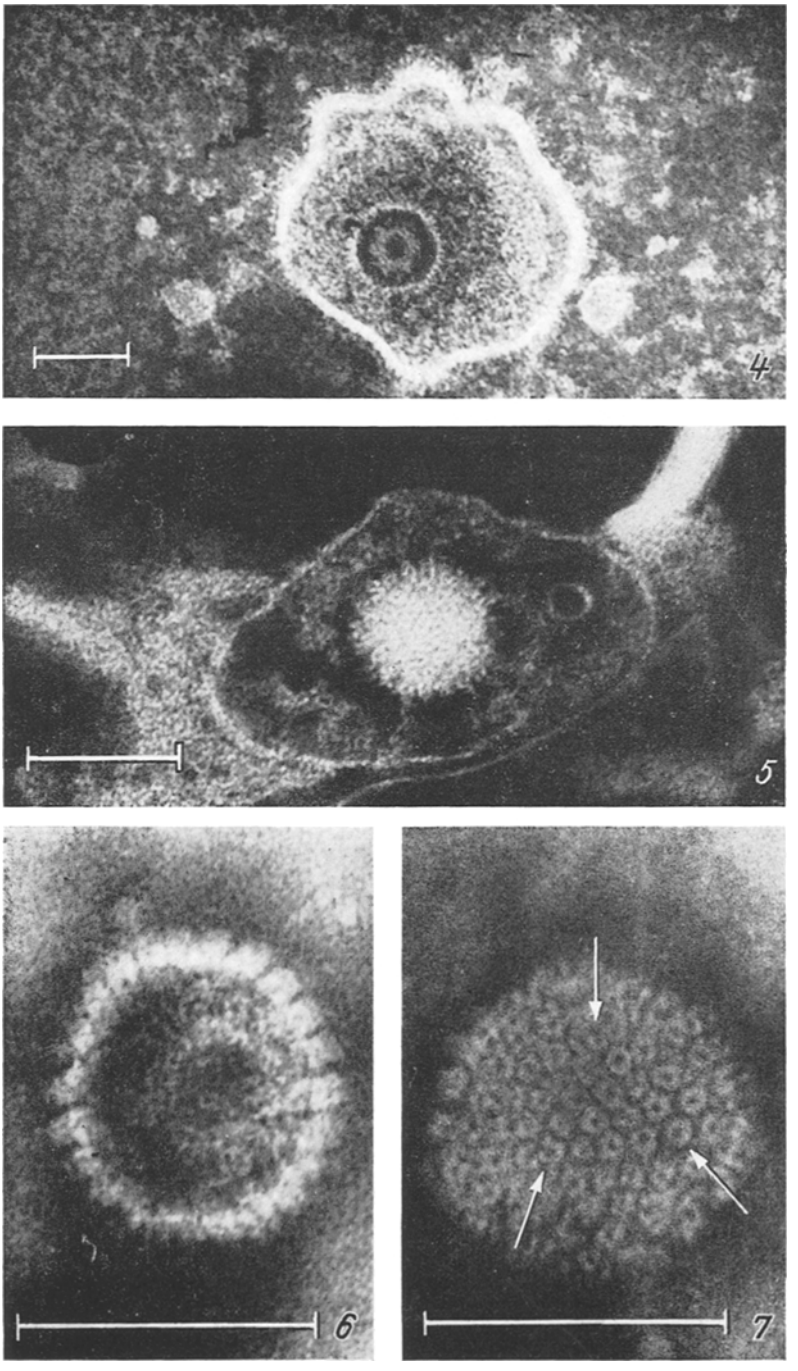

Plate 2. PTA-negatively stained electron-micrographs of herpesvirus strigis virions. Bars = 100 nm

As expected, size and form of envelopes varied greatly (Figs. 3, 4 and 5). Quite frequently, spike-like projections were visible, forming fringes at the outer contour of the envelope (Fig. 4).

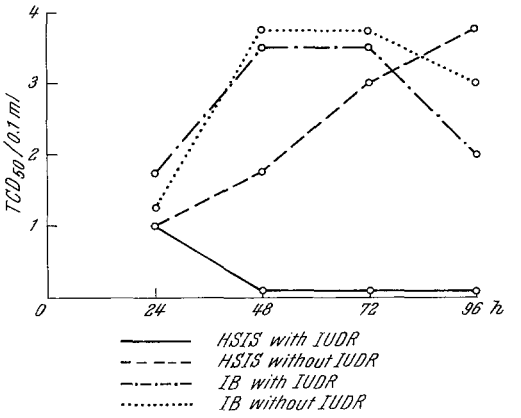

Fig. 8

4. Discussion

Herpesvirus strigis was shown to possess a number of biochemical and biophysical properties compatible with its classification into the genus herpesvirus. Its replication was suppressed by IUDR, indicating its essential nucleic acid is DNA. It proved sensitive to ether, chloroform and trypsin. It was labile towards low pH and heat. All these features are common properties of established herpesviruses. Most of these properties were also shared by a German isolate of HSIS virus (18).

The observation of naked as well as enveloped virions in our negatively stained preparations could be expected. BURTSCHER and SCHUHMACHER (10) in ultrathin sections earlier demonstrated that *herpesvirus strigis* is present in the naked form in the nuclei, acquires an envelope upon passage through the nuclear membrane [common feature of herpesvirus replication (21)], and occurs only in enveloped form in the cytoplasm. The relatively high percentage of naked virions encountered in our PTA stained preparations might be explained by the early harvest of infected cells and the slow liberation (7) of *herpesvirus strigis* from infected cells.

With an average diameter of 100 nm for naked and of 160 to 250 nm for enveloped HSIS virions our values are in good agreement with those determined

Fig. 4. Enveloped virus particle containing a capsid with a bright core with a dark center. Spike-like projections surround almost the entire envelope

Fig. 5. Enveloped virion with typical herpesvirus-like hollow capsomeres

Fig. 6. Well contrasted virion displaying capsomeres as hollow cylinders and a core structure

Fig. 7. Capsid with 5 capsomeres along each edge of an equilateral triangle facet (arrows). Herpesvirus icosahedron structure and number of capsomeres may be made out

for other herpesviruses in negatively stained preparations, *i.e.* herpes simplex virus (22), pseudorabies virus (17), varicella virus (3), infectious laryngotracheitis virus (12, 20).

In ultrathin sections (10) virions of *herpesvirus strigis* measured in diameter some 20 per cent less than in negatively stained preparations. The same observation has been made with infectious laryngotracheitis virus (20), another avian herpesvirus.

In Figure 7 a *herpesvirus strigis* virion clearly displays a triangular facet with 5 capsomeres along each edge to the viewer. This facet is one of 20 in a virion with icosahedral symmetry (11). Using the formula of HORNE and WILDY (14) $x = 10(n-1)^2 + 2$, and putting $n = 5$, we arrive at a total number of 162 capsomeres, which unequivocally classifies HSIS virus as an herpesvirus (14).

Other authors had observed similar core structures with some of their herpesviruses (12, 20), and also fringes on the viral envelope (3, 12, 20), of the size recorded here for *herpesvirus strigis*. Thus structurally HSIS virus is a typical representative of the genus herpesvirus. Based on its biological properties described in our first paper (7), which allowed to differentiate it from other herpesviruses, we proposed HSIS as a new avian virus species to be named *herpesvirus strigis*.

References

1. ANDREWES, CHR., and D. M. HORSTMAN: The susceptibility of viruses to ethyl ether. J. gen. Microbiol. **3**, 290—297 (1949).
2. ANDREWES, CHR., and H. G. PEREIRA: In: Viruses of Vertebrates. 2nd Ed., p. 432. London: Baillière, Tindall and Cassell, 1967.
3. ALMEIDA, J., A. F. HOWATSON, and M. G. WILLIAMS: Morphology of varicella (chicken pox) virus. Virology **16**, 353—355 (1962).
4. BÖGEL, K., und H. BÖHM: Ein Rhinovirus des Rindes. Zbl. Bakt. I. Abt. Orig. **187**, 2—14 (1962).
5. BÖGEL, K., und A. MAYR: Untersuchungen über die Chloroformresistenz der Enteroviren des Rindes und des Schweines. Zbl. Vet.-Med. **8**, 908—922 (1961).
6. BÜRKI, F.: Studien über bovine Enteroviren. 1. Mitteilung. Zbl. Vet.-Med. **9**, 748 to 759 (1962).
7. BÜRKI, F., H. BURTSCHER, and M. SIBALIN: Herpesvirus strigis, a new avian species of the genus herpesvirus. I. Biological properties. Arch. ges. Virusforsch. **43**, 14—24 (1973).
8. BÜRKI, F., and L. PICHLER: Further biochemical testing of feline picornaviruses. Arch. ges. Virusforsch. **33**, 126—133 (1971).
9. BURTSCHER, H.: Die virusbedingte Hepatosplenitis infectiosa strigum. 1. Mitteilung: Morphologische Untersuchungen. Path. Vet. **2**, 227—255 (1965).
10. BURTSCHER, H., und A. SCHUMACHER: Morphologische Untersuchungen zur Virusätiologie der Hepatosplenitis infectiosa strigum. Path. Vet. **3**, 506—528 (1966).
11. CASPAR, D. L. D., and A. KLUG: Physical principles in the construction of regular viruses. Cold Spr. Harb. Symp. quant. Biol. **27**, 1—24 (1962).
12. CRUICKSHANK, J. G., D. M. BERRY, and B. HAY: The fine structure of infectious laryngotracheitis virus. Virology **20**, 376—378 (1963).
13. DINTER, Z.: Relationship between bovine virus diarrhea virus and hog cholera virus. Zbl. Bakt. I. Abt. Orig. **188**, 475—486 (1963).
14. HORNE, R. W., and P. WILDY: Symmetry in virus architecture. Virology **15**, 348 to 373 (1961).
15. MILLONIG, G.: Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. appl. Physics **32**, 1637 (1961).

16. Reed, L. J., and H. A. Muench: A simple method of estimating fifty percent endpoints. Amer. J. Hyg. **27**, 493—497 (1938).
17. Reissig, M., and A. S. Kaplan: The morphology of noninfective pseudorabies virus produced by cells treated with 5-fluorouracil. Virology **16**, 1—8 (1962).
18. Schettler, C. H.: *In vitro* Untersuchungen über die Eigenschaften des Virus der Hepatitis et Splenitis Infectiosa Strigum. In: Erkrankungen der Zootiere, pp. 205 to 209. Berlin: Akademie Verlag, 1970.
19. Schneweis, K. E.: Über die Haltbarkeit von drei Herpes simplex Virusstämmen bei tiefen Temperaturen. Z. Hyg. Infect.-Kr. **147**, 319—326 (1961).
20. Watrach, A. M., L. E. Hanson, and M. A. Watrach: The structure of infectious laryngotracheitis virus. Virology **21**, 601—608 (1963).
21. Wildy, P.: Classification and nomenclature of viruses. Monographs in Virol. Vol. 5, p. 81. Basel-München-Paris-London-New York-Sidney: S. Karger, 1971.
22. Wildy, P., W. C. Russell, and R. W. Horne: The morphology of herpesvirus. Virology **12**, 204—222 (1960).
23. Zwillenberg, L. O., and F. Bürki: On the capsid structure of small feline and bovine RNA viruses. Arch. ges. Virusforsch. **19**, 373—384 (1966).

Authors' address: Dr. M. Sibalin, Institute of Virology, Veterinary University, Linke Bahngasse 11, A-1030 Wien, Austria.