Comparison of 6 different reoviruses of various reptiles

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Vet Res (1995) 26, 470-476

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Summary — In the last years the number of reports on virus isolations from reptiles have increased. The relationship of reptilian viruses to mammalian and avian viruses has not been fully investigated to date. In this paper 6 reptilian reoviruses have been examined and compared with avian and mammalian reoviruses with respect to serological and physicochemical properties. Differences and similarities are described.

reovirus / reptile / avian reovirus

Résumé — Comparaison de 6 réovirus provenant de différents reptiles. *Ces dernières années, de nombreuses études ont rapporté l'isolement de virus chez des reptiles. Les relations entre les virus de reptiles et les virus de mammifères et d'oiseaux n'ont pas été très étudiées jusqu'à présent. Nous avons examiné 6 réovirus de reptiles, et avons comparé leurs propriétés sérologiques et physicochimiques à celles de réovirus aviaires et de mammifères. Les similitudes et les différences sont exposées ici.*

réovirus / reptile / réovirus aviaire

INTRODUCTION

Viruses in reptiles have become more and more important in the last years. The subjects of the first studies in reptiles were arthropod-borne viruses and the aim was to investigate the transmission on these viruses *via* reptiles. Prior to 1970, little was known about the problem of viral diseases in reptiles themselves.

One of the first recognized diseases in reptiles which seemed to be caused by a viral agent was the paramyxovirus infection in snakes (Fölsch and Leloup, 1976). Several other groups of viruses have now been detected in reptiles (Ahne, 1991). Their pathogenic potential has not always been determined.

Isolation of reptilian viruses started with the improvement of new cell culture tech-

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niques. Using cell cultures of reptilian origin, the isolation of different groups of viruses was possible.

Raynaud and Adrian (1976) detected reovirus-like particles in the papilloma of a Green lizard during light-microscopical examination of the tissue. They also found particles of papovaviruses and herpesviruses; the importance of the reovirus particles in this case is not yet clear. Jacobson (1986) reported the isolation of reovirus from imported Chinese vipers which died shortly after acquisition. He also described a cytopathic effect in a snake cell culture with formation of giant syncytia. Electron microscopic examination of organ tissues of the diseased snakes and the infected cell culture revealed reovirus-like particles.

Ahne *et al* (1987) isolated a reovirus from a ball python. This reovirus was investigated further and compared with mammalian reoviruses. In contrast to mammalian reoviruses it did not agglutinate human erythrocytes and was not neutralized by antisera against mammalian serotypes. In cell culture it showed fusion of cells and also formation of syncytia.

Another reovirus was isolated from an Emerald tree boa (Blahak and Göbel, 1991), which was found dead in its cage. This virus revealed characteristics similar to the reovirus of the ball python, *eg*, lack of hemagglutinating activity and fusion of cells in cell culture. The results of an agar gel precipitation test of this virus indicated a serological relationship to avian reoviruses. Investigations on the preferred incubation temperature of reptilian reoviruses were reported (Blahak, 1993).

This paper compares 6 reoviruses of different reptiles. Four viruses were isolated in our laboratory. Two of them originate from snakes, the Emerald tree boa *(Corallus caninus)* reovirus mentioned above (109/90) and a reovirus of an Aesculapian snake (*Elaphe longissima,* 5327/91). The Aesculapian snake died during an outbreak of a paramyxovirus infection in a German zoo.

Two viruses were isolated from Green iguanas *(Iguana iguana).* One iguana died 2 weeks after acquisition, showing anorexia (1523/93). Two iguanas out of a group of new acquired young iguanas died 1 week after introducing them in the new cage (1118/94). The clinical signs were non-specific.

These 4 viruses were compared with a reovirus from a Ball python (*Python regius,* PR), isolated from Ahne *et al* (1987) and a reovirus from a rattle snake (*Crotalus viridis,* CV) isolated in the Institut für Veterinär-Pathologie, Gießen. This reovirus was characterized by Vieler *et al* (1994).

MATERIALS AND METHODS

Isolation procedure

Small tissue samples were obtained from several organs. They were investigated at 28°C in primary cell cultures of reptilian origin and cell lines obtained from the American type culture collection (VH2; ATCC CCL 140) as described previously (Blahak *et al,* 1991).

Purification

The isolates were purified after treatment with chloroform by endpoint titrations of infectious cell culture fluid in 10-fold dilutions in microtiter plates (96-wells). The well that only showed 1 plaque was harvested and the procedure was repeated. The harvest was multiplicated in cell culture and used for virus characterization.

5-Iodo-2-deoxyuridine (IUDR) and chloroform testing

Infectious cell culture fluid was treated with 10% chloroform as described before (Blahak *et al,*

1991). IUDR (50 μg/ml) was added to the maintenance medium of an infected cell culture and the cells were examined for the occurrence of cytopathic effect as mentioned before (Blahak *et al*, 1991).

Electron microscopic examination

Electron microscopic examination of infectious cell culture fluid was done by W Herbst and his colleagues at the Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Gießen, Germany.

Hemagglutination test

The test was performed according to standard procedures with cell culture fluid in round-bottomed microtiter plates at room temperature, using serial doubling dilutions of infectious cell culture fluid (0.05 ml), 0.025 ml NaCl solution, 1% human red blood cells type 0 (0.05 ml) and 1% chicken erythrocytes.

Purification and concentration of virus particles using polyethylene glycol

Infectious cell culture fluid was purified and concentrated using polyethylene glycol in a special buffer system (Mayr *et al*, 1977).

Preparation of antisera in rabbits

After purification and concentration of each of the 6 virus suspensions, rabbits were inoculated with virus. Virus suspensions had titers of $10^{4.5}$ to $10^{6.5}$ $TCID_{50}$ per ml. The animals were boostered after 4 weeks and bled 2 weeks after the last booster. Serum was collected and stored at –20°C.

Cross-neutralization test

Antisera heated to 56°C for 30 min were serially diluted 2-fold with cell culture medium in microtitre plates. An equal volume of virus suspension containing 100 $TCID_{50}$ per ml was added to each well. The plates were processed as described by Mayr *et al* (1977). Plates were read after 7 d, taking the highest serum dilution which showed total virus neutralization as the antibody titres.

Preparation of samples for electrophoresis

This was performed as described by Vieler *et al* (1994).

Polyacrylamide gel electrophoresis

Electrophoresis was performed according to the method of Laemmli (1970) in a 7.5% discontinuous polyacrylamide gel.

Visualization of results

Results of the electrophoresis were visualized with the silver stain kit, Biorad GmbH.

Reference viruses and avian antisera

Chicken reovirus strain S1133 and mammalian reovirus type 3 were used as reference viruses (Institut für Geflügelkrankheiten, Gießen, Germany). Reovirus isolate from a rattle snake (*Crotalus viridis*, CV) and the isolate from a Ball python (Python regius, PR) were obtained by the courtesy of one of the authors (EV) and W Ahne, Institut für Zoologie, Fischereibiologie und Fischkrankheiten, Munich, Germany.

Antisera against various strains of chicken reoviruses (H 960 and S 1133), and turkey reoviruses (P 853 and P 1837), against 3 strains of psittacines (budgerigar WS 6311, Grey parrot GP58A, EP10), one cockatoo isolate (Palm cockatoo PK 857) and one isolate of *Anhima cornuta* (WV 4445/46) were produced in the Institut für Geflügelkrankheiten, Gießen, Germany.

RESULTS

Isolation of virus succeeded at 28°C in viper heart cells (VH2, ATCC CCL N° 140) and a primary culture of tortoise fibroblasts in the case of the Emerald tree boa isolate. Preliminary changes in the cell culture could be noticed after 3–5 d. The cytopathic effect started with granulation and fusion of the cells and led to formation of giant syncytia which detached from the monolayer (table I).

Supernatants of positive cell cultures were tested for their kind of nucleic acid using the IUDR test. Adding IUDR to the maintenance medium of an infected cell culture showed no effect on the replication of the 4 viruses. Treatment of infectious supernatant with chloroform did not inhibit the development of cytopathic effect in any of the 4 isolates.

The results of the 2 tests indicate that the isolates are non-enveloped viruses with RNA as the type of nucleic acid. Electron microscopic examination of infectious fluids revealed the presence of reovirus particles. Concomitant viruses were not detected. Tests for hemagglutinating activity using 1% human erythrocytes and 1% chicken erythrocytes were negative for all isolates.

Serological relationship was determined with a cross-neutralization test. Antisera prepared against all isolates were tested with the 6 reovirus antigens (table II).

Cross-reactions could be detected and were confirmed using the formula of Archetti and Horsfall (1950). The resulting *r* values are reported in table II. One-way neutralization tests with antisera against different serotypes of avian reoviruses (see *Materials and methods)* and against the mammalian serotype 3 were performed. No inhibition of virus multiplication could be observed.

The results of an electrophoretic analysis of the viral RNA were visualized with silver staining. The 6 reptilian reoviruses were compared with a chicken reovirus and mammalian reovirus type 3. They revealed 10 segments of RNA, which could be separated according to their migrating distances into 3 size classes. The migration pattern resembled the pattern of the chicken reovirus. However, some differences could be noticed. The S1 segment was clearly distinguishable from the S2, S3 and S4 segments, but it migrated nearly midway between the M and S size class whereas the S1 segment of the chicken reovirus was situated closer to the M class.

The 6 reptilian viruses exhibited only minor differences between themselves. The S1 segment of the CV migrated closer to the M class in comparison to the other viruses. The 3 segments of the M class did

Table I. Time taken after infection (d) for first cytopathic effects.

	Emerald tree boa (109/90)	*Aesculapian snake (5327/91)*	*Green iguana (1523/93)*	*Green iguana (1118/94)*
Lung	Negative	Negative	ne	Negative
Liver	Negative	Negative	3	5
Spleen	ne	ne	3	Negative
Kidney	5	4	ne	ne
Intestine	15	3	Negative	5

Negative = the sample remained negative after 2 passages in viper heart cells; ne = not examined.

Table II. Cross-neutralization tests. Titres determined as $\log_2$ using 100 $TCID_{50}$ of virus suspension. In parentheses, *r* value: the degree of relatedness according to Archetti and Horsfall.

Isolate	*Antisera*					
	109/90	*5327/91*	*1523/93*	*1118/94*	*PR*	*CV*
109/90	11.5 (1.0)	4.0 (0.43)	11.5 (0.97)	< 0.5 (0.01)	10.5 (0.98)	< 0.5 (< 0.01)
5327/91	6.0 (0.43)	12.0 (1.0)	6.0 (< 0.01)	< 0.5 (< 0.01)	8.5 (< 0.01)	< 0.5 (< 0.01)
1523/93	8.0 (0.97)	< 0.5 (< 0.01)	8.5 (1.0)	< 0.5 (< 0.01)	8.5 (1.0)	< 0.5 (< 0.01)
1118/94	< 0.5 (< 0.01)	7.0 (< 0.01)	6.5 (< 0.01)	10.5 (1.0)	< 0.5 (< 0.01)	< 0.5 (< 0.01)
PR	9.0 (0.98)	< 0.5 (< 0.01)	9.0 (1.0)	< 0.5 (< 0.01)	9.0 (1.0)	< 0.5 (< 0.01)
CV	9.0 (< 0.01)	11.5 (< 0.01)	7.5 (< 0.01)	< 0.5 (< 0.01)	7.5 (< 0.01)	11.5 (1.0)

not migrate as far as the M class of the chicken reovirus and the pattern differed between the isolates.

The L class seemed to be very similar with only the L1 segment of 109/90 showing some difference.

DISCUSSION

The 4 reptilian isolates showed an insensitivity to treatment with IUDR and chloroform, indicating RNA as the type of nucleic acid and the lack of an envelope. Electron microscopic examination of infectious cell culture fluid confirmed the presence of reovirus particles. The cytopathic effect was characterized by the formation of giant syncytia leading to destruction of the monolayer after 3–5 d. This type of cytopathic effect is characteristic for avian reoviruses, as is the lack of hemagglutinating activity (Robertson and Wilcox, 1986).

On the basis of the results of the cross-neutralization test between the 6 reptilian reoviruses, relatedness values were calculated using the formula of Archetti and Horsfall (1950). The *r* values were evaluated according to the method of Brooksby (1967), recognizing a serotype difference if the *r* value was between 0 and 0.1, a major subtype difference if the *r* value was between 0.1 and 0.32, a minor subtype difference if the *r* value was between 0.32 and 0.7, and little or no difference if the *r* value was greater than 0.7.

The *r* values revealed a close relationship between the isolate of the Emerald tree boa (109/90), the iguana isolate (1523/93) and the isolate of the Ball python (PR). Only a minor subtype variation could be detected between the isolate of the Aesculapian snake (5327/91) and the isolate of the Emerald tree boa (109/90) according to the method of Brooksby (1967). The iguana isolate (1118/94) and the rattle snake isolate (CV) revealed no serological relationship to any of the other reptilian isolates. According to the results, the 6 viruses may be grouped into 3 serotypes:

1) The isolate of the Emerald tree boa (109/90), one iguana isolate (1523/93) and the isolate of the Ball python (PR); the isolate of the Aesculapian snake (5327/91) showed some minor variations but seemed to be a member of the first serotype.

2) The iguana isolate (1118/94), which showed some 1-way reactions with antisera against the isolates 1523/93 and 5327/91.

3) The rattle snake isolate (CV) which exhibited some 1-way reactions with antisera against 109/90, 5327/91, PR and 1523/93.

There were, however, some 1-way reactions between the isolate of the rattle snake and antisera against the isolates of the Emerald tree boa (109/90), the Aesculapian snake (5327/91), the Ball python (PR) and the iguana isolate (1523/93) (table II). Similar 1-way neutralization could be observed between the iguana isolate (1118/94) and antisera against the other iguana isolate (1523/93) and the isolate of the Aesculapian snake (5327/91). This kind of 1-way neutralization is not reflected in the *r* values and may give reason for a cautious interpretation of the degree of antigenic relatedness (Robertson and Wilcox, 1984).

All isolates could be differentiated from the avian and mammalian reoviruses used in this test, according to the results of the one-way neutralization test. However, considering the results of the cross-neutralization tests between the 6 reptilian isolates, 1-way reactions may exist between the antisera against the reptilian viruses and the avian reoviruses. This should be tested in the near future.

In contrast to these results, an agar gel precipitation test performed with the Emerald tree boa isolate and antiserum against chicken reovirus S1133 showed a clear precipitation line (Blahak and Göbel, 1991). Bearing in mind that the reptilian isolates exhibit a similar type of cytopathic effect in cell culture and lack hemagglutinating activity, one may speculate that at least some of the reptilian reoviruses share the group specific antigen with avian reoviruses.

Similar results were reported by Heffels-Redmann *et al* (1992) in their publication about characterization of reoviruses from Muscovy ducks. The Muscovy duck reoviruses share the group specific antigen with chicken reoviruses but are not inhibited by antiserum against chicken reovirus. The heterogeneity of avian reoviruses has been documented by several authors (Wood *et al,* 1980; Hieronymus *et al,* 1983; Robertson and Wilcox, 1986). A possible explanation could be the nature of the neutralizing kinetics of reoviruses. Three neutralizing epitopes, 1 type-specific and 2 group-specific have been detected in avian reoviruses (Takehara *et al,* 1987). Polyclonal antisera may be directed against either epitope. The same mechanism may work in reptilian reoviruses and may explain some of the 1-way cross reactions and the difficulties in grouping the isolates.

The electrophoretic analysis of the RNA revealed a similar migration pattern to the chicken reovirus. Some minor differences could be detected between the chicken reovirus and between the reptilian reoviruses themselves. Polymorphism of the migration pattern even between viruses of the same serotype is well documented in avian reoviruses (Robertson and Wilcox, 1986; Heffels-Redmann *et al,* 1992). Therefore the results of the RNA preparation substantiate the hypothesis of a close relationship between avian and reptilian reoviruses.

Further investigations concerning the relationship between reptilian and avian reoviruses seem to be necessary and should include sequencing and comparison of the genome of the reptilian reoviruses.

ACKNOWLEDGMENT

The authors are grateful to W Ahne for his isolate of a *Python regius* (PR).

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