

# A Serosurvey of Borna Disease Virus Infection in Wild Rats by a Capture ELISA

Koji TSUJIMURA, Tetsuya MIZUTANI\*, Hiroaki KARIWA, Kumiko YOSHIMATSU<sup>1)</sup>, Michiko OGINO<sup>1)</sup>, Yuko MORII<sup>1)</sup>, Hisae INAGAKI, Jiro ARIKAWA<sup>1)</sup> and Ikuo TAKASHIMA

Laboratory of Public Health, Department of Environmental Veterinary Sciences, The Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818 and <sup>1)</sup>Institute for Animal Experimentation, School of Medicine, Hokkaido University, Sapporo 060-8638, Japan

(Received 9 July 1998/Accepted 6 October 1998)

**ABSTRACT.** For a serological diagnostic test for Borna disease (BD), we developed a capture ELISA with specificity and sensitivity based on detection of antibodies against BD virus (BDV) p40 protein. Using our capture ELISA system, the antibody response of rats inoculated intracerebrally with BDV at 4 weeks after birth showed a sharp increase from 1 to 4 weeks postinoculation (p.i.) and a steady level after 5 weeks p.i. To investigate prevalence of BDV infection among wild rats, we examined sera of *Rattus norvegicus* in Kami-iso town, Oshima district, Hokkaido, suggesting that rats in this area had not been infected by BDV.—**KEY WORDS:** Borna disease virus, capture ELISA, *Rattus norvegicus*.

*J. Vet. Med. Sci.* 61(2): 113–117, 1999

Borna disease (BD) in horses has been known as a chronic encephalomyelitis endemic in Germany and several other European countries [6]. Natural infection of BD virus (BDV) has been reported in other vertebrates; sheep, cattle, cats, rabbits and ostriches [19]. Furthermore, accumulated epidemiological data suggest the association of BDV with certain psychiatric disorders in humans [2, 5, 18], raising concerns of a potential zoonosis. Further epidemiological information is needed to understand the origin of BDV in humans and the modes of viral transmission.

BDV contains a nonsegmented negative-sense 8.9 kilobase, single-stranded RNA genome, containing at least six open reading frames encoding proteins of 40 kDa (p40), 23 kDa (p24), 10 kDa (p10), 16 kDa (gp18), 57 kDa (G) and 190 kDa (pol) [3, 4, 20–22]. The p40 protein, which is a candidate for nucleocapsid protein, is detected in high concentrations in BDV-infected animals and cultured cells [1]. Anti-p40 and p24 antibodies are easily induced in infected animals [12, 13], suggesting that p40 and p24 are suitable as diagnostic antigens for detection of serum antibodies.

Indirect immunofluorescent antibody assay (IFA) is the most widely accepted and commonly used for a practical diagnostic tool. However, IFA is not suitable for seroepizootiological surveys to handle many samples. Enzyme-linked immunosorbent assay (ELISA) is also commonly used as a practical serological diagnostic tool. Since the color development is measured with a microplate reader, many samples can be handled by ELISA. It is important for the ELISA system to use purified antigens [8], because non-specific reactions are apt to occur in the

study of BDV. Recently, we reported that, using IFA, recombinant p40 antigen produced by a baculovirus vector system was more sensitive than p24 for detection of antibodies in BDV-experimentally infected rats [17].

In this study, we obtained monoclonal antibodies (MAbs) against BDV p40 protein and used them to develop a capture ELISA system which is simple and highly specific. Using the capture ELISA system, sera from two kinds of *Rattus norvegicus* groups, experimentally infected laboratory rats and wild rats, were examined for anti-BDV antibodies.

## MATERIALS AND METHODS

**Cells and Virus:** Madin-Darby canine kidney (MDCK) cell line and persistently BDV-infected cell line (MDCK/BDV) [7] were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum.

**p40 protein expression system:** cDNA of BDV-p40 was inserted in pGEX containing GST gene (pGEX-p40), which were provided by Dr. K. Ikuta (Institute of Immunological Sciences, Hokkaido University) [15]. The p40 protein was produced in *E. coli* JM109 by recombinant DNA technology and purified by affinity chromatography using Glutathione Sepharose 4B (Pharmacia Biotech, Sweden). The purity of GST fusion p40 protein (GST-p40) was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

cDNA of p40 was recombined to a baculovirus plasmid (pBac-p40) using BAC-To-BAC baculovirus expression system (Gibco BRL, USA) [17]. The p40 protein was obtained from pBac-p40 transfected High Five cells and their supernatant.

**Production of MAbs to p40:** BALB/c mice were immunized by intramuscular injection of GST-p40 proteins (200 µg/ml) emulsified in an equal volume of Freund's complete adjuvant. Two subsequent immunizations were

\* CORRESPONDENCE TO: MIZUTANI, T. Laboratory of Public Health, Department of Environmental Veterinary Science, The Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

given at 2 week intervals. Spleen cells were collected and cell-fusion technique was performed to obtain hybridoma producing anti-p40 monoclonal antibodies. The reactivities of antibodies were tested with recombinant p40 in High Five cells produced by the baculovirus expression system (High Five/rBac-p40) using IFA [17]. Hybridomas were recloned 3 times by limiting dilution. Established hybridomas were injected into the peritoneal cavities of pristane-treated BALB/c mice to obtain ascites fluid. A protein G column (Pharmacia Biotech, USA) was used for affinity purification of MAbs from ascites fluid. Immunoglobulin classes and subclasses of the antibody were determined by means of a mouse mono Ab-ID kit (Zymed, USA).

**SDS-PAGE and immunoblotting:** SDS-PAGE was performed under reducing conditions with 5% stacking gels and 12% separating gels [11]. After electrophoresis, proteins were electrophoretically transferred to PVDF membrane (Millipore, USA). The membrane was blocked with 3% BSA in PBS for 60 min and then incubated with MAb for 60 min. After washing with PBS containing Tween 20, the membrane was incubated with horseradish peroxidase-conjugated goat-anti mouse IgG for 60 min and washed again. The membrane was incubated with substrate solution (0.25 mg/ml 4-chloro-1-naphthol, 17% methanol, 0.2% H<sub>2</sub>O<sub>2</sub> in PBS).

**Capture ELISA:** Microtiter plates were coated with 50 µl of MAb (1 µg/ml) per well and incubated at 37°C for 60 min. After washing with buffer 1 (PBS containing 0.05% Tween 20) for 3 times, the wells were blocked with 3% BSA in PBS at 37°C for 60 min and then washed 3 times. The supernatant of rBAC-p40 was diluted at 1:16, and added to the plate with the volume of 50 µl per well and then incubated at 4°C for 15 hr. After washing the plate with buffer 1, 50 µl serum samples diluted at 1:50 in buffer 2 (PBS containing 0.05% Tween 20 and 0.5% BSA) were added to the plates. The plates were incubated at 37°C for 60 min. After washing with buffer 1, the wells were incubated with 50 µl horseradish peroxidase-labelled rabbit anti-rat IgG diluted at 1:2,000 in buffer 2 at 37°C for 60 min. The washing was repeated and the wells were incubated with 100 µl of substrate solution (0.1% w/w O-phenylene-diamine dihydrochloride, 0.04% v/v H<sub>2</sub>O<sub>2</sub>) for

30 min. The color development was measured (OD 490 nm) with a microplate reader. The absorbance of each sample was calculated by subtracting the absorption value with supernatant of baculovirus mock-infected High Five cells, from that of the infected cells.

## RESULTS

**Characterization of MAbs:** Eight hybridoma clones producing MAb (B8, B9, B12, E4, E7, F3, G1 and G4) against BDV p40 recombinant protein were obtained by screening IFA assay. These MAbs did not react with the recombinant BDV p24 protein. All eight MAbs reacted with native p40 protein in BDV-persistently infected MDCK (MDCK/BDV) cells (data not shown). Most hybridoma-culture supernatants and mice ascites showed the respective IFA titers of >1:40 and >1:100,000 both to High Five/rBac-p40 and MDCK/BDV (Table 1). B9 and E7 MAbs were classified as IgG2a and the others were IgG1. Interestingly, only the B9 and E7 MAbs reacted with the protein band at approximately 40 kDa on immunoblotting following SDS-PAGE under reducing condition of MDCK/BDV cells (Fig. 1). This result suggested that B9 and E7 MAbs recognized linear epitopes of p40 protein and the other MAbs recognized conformational epitopes of p40.

**Detection of p40 protein in BDV-infected rats:** To examine detection of p40 protein *in vivo* using B9 and E7 MAbs, Lewis rats, at 24 hr after birth, were inoculated intracerebrally with homogenized MDCK/BDV cells. Rats were sacrificed at 6 weeks postinoculation (p.i.) and then immunoblotting was performed. Using the B9 and E7 MAbs, p40 protein from the brain was detected (Fig. 2).

**Reactivity of capture ELISA:** To select an MAb suitable for capture ELISA, eight MAbs were coated on microplates. As antigen, p40 recombinant protein from the supernatant of High Five cells was applied. BDV-infected rat serum at 26 weeks p.i. was diluted at 1:50 and added to capture ELISA plate. Because specific absorbance was not obtained, E7 MAb was not appropriate as a capture antibody (Fig. 3). The other MAbs except E4 and F3 never showed significant reactions with p40 antigen at the concentration of 1 µg/ml despite the positive reactions at 16 µg/ml. Since E4 MAb reacted stably at concentrations from 1 to 16 µg/ml, we

Table 1. Reactivities of anti-p40 monoclonal antibodies

MAb	subclass	High Five/rBACp40		High Five/rBACp24		MDCK/BDV		MDCK	
		culture sup.	ascites	culture sup.	ascites	culture sup.	ascites	culture sup.	ascites
B9	IgG2a	>40	>100,000	<1	NT <sup>a)</sup>	>40	>100,000	<1	NT
E7	IgG2a	>40	>100,000	<1	NT	>40	>100,000	<1	NT
E4	IgG1	>40	>100,000	<1	NT	>40	>100,000	<1	NT
G4	IgG1	>40	>100,000	<1	NT	>40	>100,000	<1	NT
B8	IgG1	>40	>100,000	<1	NT	>40	>100,000	<1	NT
F3	IgG1	>40	64,000	<1	NT	>40	64,000	<1	NT
B12	IgG1	20	>100,000	<1	NT	>40	>100,000	<1	NT
G1	IgG1	>40	>100,000	<1	NT	40	>100,000	<1	NT

<sup>a)</sup> NT: not tested

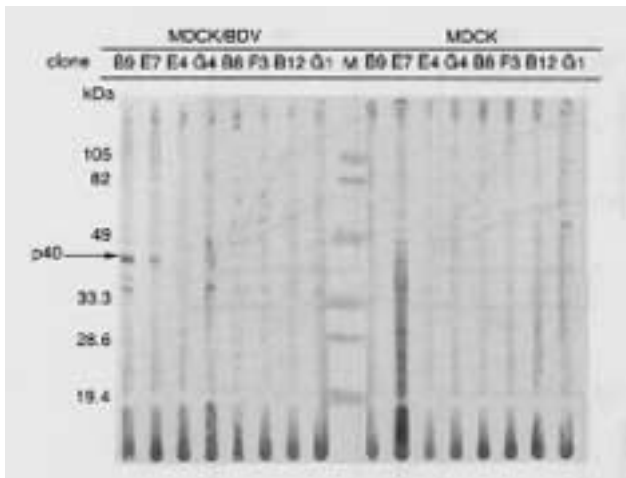


Fig. 1. Detection of p40 protein by Western blotting. The p40 in MDCK/BDV cells was detected by B9 and E7 MAbs. Molecular weights of marker proteins are indicated (lane M).

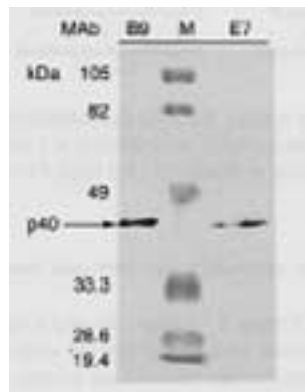


Fig. 2. Western blotting analysis using *in vivo* samples. Brain samples were obtained from adult rats inoculated with homogenized MDCK/BDV cells at 6 weeks p.i. Western blotting analysis was performed using B9 and E7. Molecular weights of marker proteins are indicated (lane M).

determined that E4 MAb was a suitable capture antibody.

**Detection of anti-p40 antibodies in experimentally BDV-infected rats:** To examine development of anti-p40 antibodies *in vivo* after inoculation with BDV, Lewis rats at 4 weeks of age were inoculated intracerebrally with homogenized MDCK/BDV cells and serum was obtained every week. As shown in Fig. 4, there is a sharp increase from 1 to 4 weeks p.i. After 5 weeks p.i., antibody levels were steady at the OD value of 0.7 (490 nm).

**Screening of *Rattus norvegicus* rats in the field:** We examined the sera of *Rattus norvegicus* rats for anti-p40 antibodies which were capture at Kami-iso town, Oshima district, Hokkaido. Compared with the antibody titers of positive controls, experimentally BDV-infected rats from 4 to 26 weeks p.i., the absorbance values of all 106 *Rattus*

*norvegicus* rats were low and ranged from – 0.139 to 0.098 (OD 490nm) (Fig. 5). The results suggested that *Rattus norvegicus* rats in this area did not have antibody against BDV-p40.

## DISCUSSION

In this study, we developed a capture ELISA system for specific detection of anti-BDV p40 antibody in experimentally infected rats and applied it to wild rats. Recently, we demonstrated that recombinant p40 protein using a baculovirus vector system was more sensitive than p24 to detect polyclonal antibodies in BDV-experimentally infected rats [17]. Therefore, we established hybridomas producing MAbs against recombinant p40 protein using a bacterial expression system. MAb B9 and E7 may recognize linear epitopes, while the other MAbs might recognize conformational epitopes. Although it was reported that anti-p40 MAb showed crossreactivity to p24 protein [10], our anti-p40 MAbs in this study reacted to only p40, not p24 protein.

The capture ELISA developed in this study was found to be specific and sensitive. None of the rats before inoculation had high absorbance values for p40. Anti-p40 antibodies were detected as early as 2 weeks p.i. and reached a plateau at 5 weeks p.i. The above results were consistent with development patterns of IFA antibody in infected rats of our previous study [17].

High prevalences of BDV infection have been demonstrated in apparently healthy animals [19]. Serological data and molecular epidemiological studies indicate that BDV can infect humans and is possibly associated with certain neuropsychiatric disorders [2, 5, 18]. Recently, a high rate of BDV seroprevalence and viral RNA positivity in peripheral blood mononuclear cells have been shown in blood samples from Japanese patients diagnosed with chronic fatigue syndrome [9, 16]. Horizontal transmission from ostriches to humans has been suggested by a seroepidemiological study of BDV infection in Israel [14, 23]. However, the reservoirs of BDV have not been clear. Since rats are highly sensitive to BDV infection experimentally, rats may be a candidate as one of the reservoirs. Although most adult rats inoculated with BDV showed no clinical symptoms, anti-p40 antibodies were detected by IFA at least 13 weeks p.i. [17], indicating that BDV infected persistently. Similar results were obtained using our capture ELISA system. Although sera from one hundred and six wild rats were examined using capture ELISA, we don't have a clear evidence that rats in this area have specific antibodies to BDV p40. More extensive seroepizootiological surveys should allow us to determine whether rodents are a reservoir of BDV. Such a study is now underway. Since there has been no standard serological diagnostic method for BDV infection, the capture ELISA system developed in this study will be useful for specific detection of anti-BDV antibody in rats and other animal species.

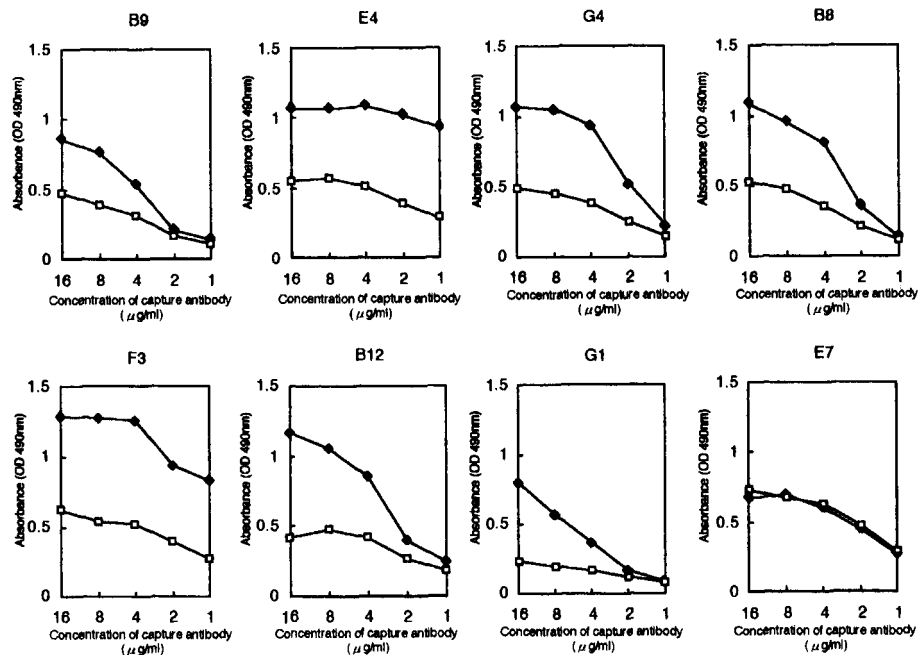


Fig. 3. Reactions of MAbs as capture antibody in the capture ELISA system. Each capture antibody was used at the concentration 1 to 16  $\mu\text{g/ml}$ . The antigen and second antibody were diluted at 1:16 and 1:2,000, respectively.  $\bullet$ : secreted rBAC p40,  $\square$ : culture medium of mock infected High Five cells.

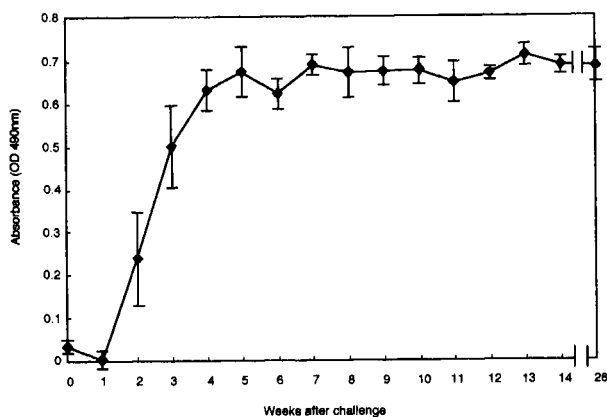


Fig. 4. Developing pattern of anti-p40 antibody response in BDV-experimentally infected rats. Capture ELISA to detect BDV antibody in infected rats was performed using E4 MAb.

**ACKNOWLEDGEMENTS.** We thank Drs. R. Rott and S. Herzog (Justus-Liebig-Universität Giessen, Giessen, Germany) for providing MDCK/BDV. We also thank Drs. K. Ikuta, T. Nakaya and M. Kishi (Immunological Science, Hokkaido University, Sapporo, Japan) for providing the plasmid pGEX-p40 and useful advice.

#### REFERENCES

1. Bause, N.I., Pauli, G. and Ludwig, H. 1991. Borna disease virus-specific antigen: two different proteins identified by monoclonal antibodies. *Vet. Immunol. Immunopath.* 27: 293–301.
2. Bode, L., Riegel, S., Lange, W. and Ludwig, H. 1992. Human infections with Borna disease virus: seroprevalence in patients with chronic diseases and healthy individuals. *J. Med. Virol.* 36: 309–315.
3. Briese, T., Schneemann, A., Lewis, A. J., Park, Y. S., Kim, S., Ludwig, H. and Lipkin, W. I. 1994. Genomic organization of Borna disease virus. *Proc. Natl. Acad. Sci. USA* 91: 4362–4366.
4. Cubitt, B., Oldstone, C. and de la Torre, J. C. 1994. Sequence and genome organization of Borna disease virus. *J. Virol.* 68: 1382–1396.
5. de la Torre, J. C., Gonzales, D. D., Cubitt, B., Mallory, M., Mueller, L. N., Grasser, F.A., Hansen, L. A. and Masliah, E. 1996. Detection of Borna disease virus antigen and RNA in human autopsy brain samples from neuropsychiatric patients. *Virology* 223: 272–282.
6. Gellert, M. 1995. "In the beginning the horse is sad"—A historical abstract of Borna disease. *Tierärztliche Praxis.* 23: 207–216.
7. Herzog, S. and Rott, R. 1980. Replication of Borna disease virus in cell cultures. *Med. Microbiol. Immunol.* 168: 153–158.
8. Horimoto, T., Takahashi, H., Sakaguchi, M., Horikoshi, K., Iritani, S., Kazamatsuri, H., Ikeda, K. and Tashiro, M. 1997. A reverse-type sandwich enzyme-linked immunosorbent assay for detecting antibodies to Borna disease virus. *J. Clin. Microbiol.* 35: 1661–1666.
9. Kitani, T., Kuratsune, H., Fuke, I., Nakamura, Y., Nakaya, T., Asahi, S., Tobiume, M., Yamaguti, K., Machii, T., Inagi, R., Yamanishi, K. and Ikuta, K. 1996. Possible correlation

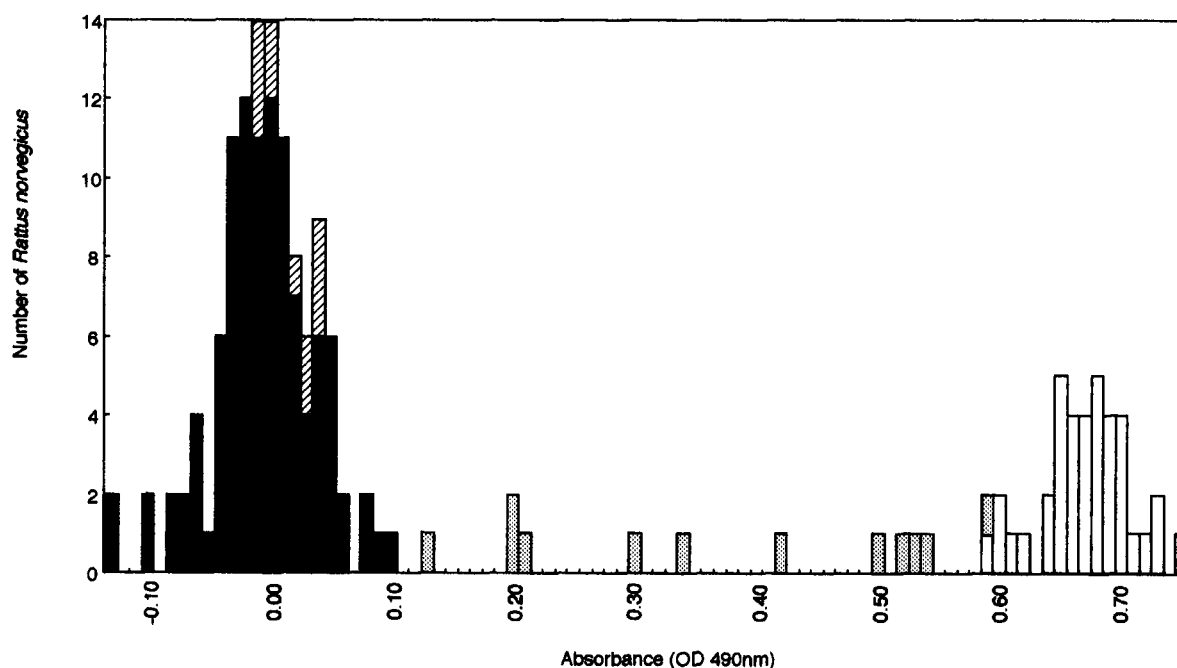


Fig. 5. Detection of anti-p40 antibody in *Rattus norvegicus* by capture ELISA system. One hundred and six *Rattus norvegicus* were examined in this study. Black column: sera from wild *Rattus norvegicus*, diagonal lines: sera from BDV-infected laboratory rats at 0–1 weeks p.i., dots: sera at 2–3 weeks p.i., blank column: sera at 4–26 weeks p.i.

- between Borna disease virus infection and Japanese patients with chronic fatigue syndrome. *Microbiol. Immunol.* 40: 459–462.
10. Kliche, S., Stitz, L., Mangalam, H., Shi, L., Binz, T., Niemann, H., Briese, T. and Lipkin, W. I. 1996. Characterization of the Borna disease virus phosphoprotein, p23. *J. Virol.* 70: 8133–8137.
  11. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
  12. Ludwig, H., Koester, V., Pauli, G. and Rott, R. 1977. The cerebrospinal fluid of rabbits infected with Borna disease virus. *Arch. Virol.* 55: 209–223.
  13. Ludwig, H. and Thein, P. 1977. Demonstration of antibodies in the central nervous system of horses naturally infected with Borna disease virus. *Med. Microbiol. Immunol.* 163: 215–226.
  14. Malkinson, M., Weisman, Y., Ashash, E., Bode, L. and Ludwig, H. 1993. Borna disease in ostriches. *Vet. Rec.* 133: 304.
  15. Nakanura, Y., Kishi, M., Nakaya, T., Asahi, S., Tanaka, H., Sentsui, H., Ikeda, K. and Ikuta, K. 1995. Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells from healthy horses in Japan. *Vaccine* 13: 1076–1079.
  16. Nakaya, T., Takahashi, H., Nakamura, Y., Asahi, S., Tobiume, M., Kuratune, H., Kitani, T., Yamanishi, K. and Ikuta, K. 1996. Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells derived from Japanese patients with chronic fatigue syndrome. *FEBS Lett.* 378: 145–149.
  17. Ogino, M., Yoshimatsu, K., Tsujimura, K., Arikawa, J., Mizutani, T. and Takashima, I. 1998. Evaluation of serological diagnosis of Borna disease virus infection using recombinant proteins in experimentally infected rats. *J. Vet. Med. Sci.* 60: 531–534.
  18. Rott, R., Herzog, S., Fleischer, B., Winokur, A., Amsterdam, J., Dyson, W. and Koprowski, H. 1985. Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. *Science* 228: 755–756.
  19. Rott, R. and Becht, H. 1995. Natural and experimental Borna disease in animals. *Curr. Top. Microbiol. Immunol.* 190: 17–30.
  20. Schneemann, A., Schneider, P., Lamb, R.A. and Lipkin, I. 1995. The remarkable coding strategy of Borna disease virus: A new member of the nonsegmented negative strand RNA viruses. *Virology* 210: 1–8.
  21. Schneider, P. A., Hatalski, C. G., Lewis, A. J. and Lipkin, W. I. 1997. Biochemical and functional analysis of the Borna disease virus G protein. *J. Virol.* 71: 331–336.
  22. Wehner, T., Ruppert, A., Herden, C., Frese, K., Becht, H. and Richt, J. A. 1997. Detection of a novel Borna disease virus-encoded 10 kDa protein in infected cells and tissues. *J. Gen. Virol.* 78: 2459–2466.
  23. Weisman, Y., Huminer, D., Malkinson, M., Meir, R., Kliche, S. and Lipkin, W. I. 1994. Borna disease virus antibodies among workers exposed to infected ostriches. *Lancet* 344: 1232–1233.