Comparison of an Enzyme-Linked Immunosorbent Assay, an Immunofluorescence Assay and a Hemagglutination Inhibition Assay for Detection of Antibodies to K-Papovavirus in Mice

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Abstract | The sensitivity of a newly developed enzyme-linked immunosorbent assay (ELISA) for detection of antibody to K virus was compared with the sensitivities of an immunofluorescence assay (IFA) and a hemagglutination inhibition assay (HIA). Specific pathogen-free BALB/c RIVM mice, 5 weeks old, were inoculated intraperitoneally with a mouse organ suspension containing $10^{4.5}$ TCID$_{50}$ of K virus per dose. Control animals were inoculated with a control mouse organ suspension. No clinical signs were observed during the 7 weeks they were followed for the development of serum antibody. The ELISA proved to be the most sensitive of the three assays and demonstrated K virus-specific antibodies as early as 3 days after infection.

K virus, a member of the family Papovaviridae (1), has been identified as a cause of asymptomatic infection in laboratory (2) and wild adult mice (3) and as a cause of severe respiratory illness in mice under 10 days of age. For both health surveillance programs of laboratory animals, and for the control of biological products, produced or controlled in rodents or rodent cells, fast and reliable serological test procedures are needed (4). Currently, two serological tests have been described for the detection of antibodies to K virus in mice, including the immunofluorescence assay (IFA) and a hemagglutination inhibition assay (HIA) (5,6).

Here, we describe an enzyme-linked immunosorbent assay (ELISA) for the serology of K virus, using a principle also employed in the serological screening for other rodent viruses (7,8), which is therefore suitable for the incorporation in automated screening programs. We infected mice intraperitoneally with K virus and followed antibody development in these animals with the three assays.

Materials and Methods

**Virus and antigen preparation:** K virus, strain Kilham, was obtained¹ and stock virus was prepared by infecting litters of SPF N:NIH/RIVM outbred mice² intracerebrally with about $10^{5.5}$ LD$_{50}$ and preparing a mixed homogenate of their lungs and livers 8 days post infection, essentially as described (9). These animals were caesarian derived, barrier-contained and certified free of K-papovavirus and other virus infections including Hantavirus (ELISA), lymphocytic choriomeningitis virus (IFA), encephalomyocarditis virus (virus neutralization assay), REO virus (ELISA), Sendai virus (ELISA), epidemic diarrhoea of infant mice (ELISA), K virus (ELISA), minute virus of mice (ELISA), mouse adenovirus (ELISA), mouse cytomegalovirus (ELISA), mouse encephalomyelitis virus (ELISA), mouse hepatitis virus (ELISA) pneumonia virus of mice (ELISA), polyoma virus (ELISA) and Toolan virus (HIA), as was shown by regular serological screening of 1 to 5% of the mice in the colony. Virus infectivity of the stock virus was determined in a TCID$_{50}$ assay on confluent monolayers of mouse embryo cells in coverslip flasks (9). After incubation for 11 days, cultures were fixed with $-70^\circ$C ethanol and examined for the presence of viral antigen by fluorescent antibody staining. For the preparation of ELISA antigen, lungs and livers of infected mice were homogenized and extracted twice for 30 seconds with 0.5 volume trichlorotrifluoroethane. The homogenate was centrifuged for 10 minutes at 1600 x g, the supernatant was layered on top of a 30 to 40% (w/v) CsCl gradient and centrifuged in a Beckman SW 50.1 rotor for 1 hour at 35 K. The virus band was collected and dialyzed against PBS (pH 7.2). ELISA antigen was prepared from stock virus by UV inactivation as described (5). The presence of papovavirus particles was confirmed by negative contrast

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EM (not shown). The protein concentration determined by the Bradford method (10) was adjusted to 600 μg/ml and 0.1 ml aliquots of this preparation were frozen at -70°C until use as antigen in ELISA.

**Virus infection and serum sampling:** Two groups of five, 5-week old SPF BALB/c RIVM mice were inoculated intraperitoneally with either 0.1 ml stock virus containing 10^6 TCID_50/ml or with a control organ suspension, respectively. The SPF status of these BALB/c RIVM mice was the same as described above for the N:NIH/RIVM mice. Blood samples were collected by orbital puncture at regular intervals up to 7 weeks post-inoculation. The serum samples were heat-inactivated and frozen at -20°C until use in the respective serological assays. A reference mouse antiserum preparation against K virus⁵ and a mouse reference antisera against polyomavirus⁶ of mice were obtained.

**Hemagglutination inhibition assay (HIA):** HIA was performed in microtiter plates⁷ essentially as described (4). Mouse sera were absorbed to sheep erythrocytes by adding 0.2 volumes of a 50% erythrocyte suspension and allowing these to settle for 2 hours, diluted 1:10 in phosphate buffered saline pH 7.2 (PBS), and titrated in serial 3-fold dilutions.

In addition, 50 sera from a SPF mouse colony⁸, free from K virus infection as shown by regular screening in HIA and IFA, were collected. The SPF status of these BALB/c RIVM mice was the same as described above for the N:NIH/RIVM mice.

**Indirect immunofluorescence (IFA):** The indirect immunofluorescence test was performed essentially as described (2) in ten well drop slides with K virus-infected and uninfected primary mouse embryo cells fixed with -70°C ethanol and stored at -70°C until use. After dilution of the mouse sera 1:30 in PBS, they were titrated further in serial 3-fold dilutions.

**Indirect enzyme-linked immunoassay:** The test was performed in polystyrene microtiter plates⁹. Fifty μl of antigen at an optimal working dilution of 1:50 (determined by block titration) in carbonate/bicarbonate buffer (pH 9.6) were added to each well and incubated for 18 hours at room temperature. Plates were washed twice for 10 seconds with tap water containing 0.05% Tween 80 and filled with 100 μl of the blocking solution PBS-BSA (PBS pH 7.2 containing 0.05% Tween 80 and 1% BSA [w/v]). After incubation for 30 minutes at 37°C, plates were washed as described above. Fifty μl aliquots of serial 3-fold dilutions of sera in PBS-BSA, with a starting dilution of 1:30, were added to each well. After incubation for 1 hour at 37°C, the plates were washed as described and 50 μl volumes of a mixed horseradish peroxidase-conjugated goat anti-mouse IgG, IgM and IgA preparations were added, incubated for 1 hour at 37°C and washed as described above. Fifty μl volumes of substrate solution (0.1mg/ml 3,3',5,5'-Tetramethylbenzidine (TMB) in 0.11M NaAc-citric acid buffer (pH 5.5) supplemented with 0.006% H_2O_2 were added, and the reaction was stopped after 10 minutes by adding 50 μl volumes of 2.0 M H_2SO_4. The absorbance was measured spectrophotometrically at 450 nm in a Tertiak Multiskan. The antibody titer of each serum sample taken from the mice experimentally infected with K virus was found by determining the reciprocal of the serum dilution at which 50% of the maximal OD450 nm (1.5) was still obtained.

**Correlation coefficients:** The correlation coefficients between data obtained with the different serological tests were calculated by standard correlation analysis (14).

**Results**

No clinical signs were observed in the experimentally infected or the control animals during the course of the experiment.

Serum samples collected from the K virus-infected and the sham-infected BALB/c RIVM mice during the 7 week period were tested in the respective serological assays. The development of anti-K virus antibody titers in the sera of the experimentally infected mice measured in ELISA, IFA and HIA is shown in Figure 1. As early as 3 days after infection, ELISA titers of 10^9 were found in the sera of all five infected animals. Titers gradually increased to 10^10 in the sera of infected animals from about 10^9 to about 10^10 at the end of the experiment. Antibodies were not detected in the HIA before 3 weeks after infection, and the geometric mean titer was about 10^3.5 at 7 weeks.

In order to determine the correlation between

![Figure 1](attachment:image.png)

**Figure 1** Development of serum antibody titers (geometric mean titers and variation) in BALB/c mice infected intraperitoneally with K virus (10^6 TCID_50) as measured in ELISA (☐-☐), IFA (☐-☐) and HIA (☐-☐).
the results obtained with individual serum samples in the respective serological assays, the data were visualized in a three dimensional plot (Figure 2). Correlation coefficients between positive results obtained in the respective assays, proved to be 0.95, 0.94 and 0.87 when comparing ELISA-IFA, ELISA-HIA and IFA-HIA, respectively.

No antibodies against K virus were detected with any of the three tests in the serum samples from the sham-infected mice, the 50 sera from the SPF mouse colony or in the reference anti-polyoma serum.

Discussion

As expected, and in contrast to the clinical signs and deaths observed in the baby mice infected intracerebrally with K virus for stock virus production, no clinical signs were observed in the adolescent BALB/c mice after intraperitoneal infection (5). However, it should be emphasized that an unnatural route of infection such as intraperitoneal inoculation may not give relevant information about clinical signs which may occur during natural infection.

The possibility of incorporating ELISA systems for routine serology of rodent viruses in automated and well standardized systems and the previously documented increased sensitivity of these assays, as compared to other serological screening systems for this purpose (7,8,11,12), have prompted us to develop an ELISA for the serology of K virus. The use of direct antigen coating to a solid phase like in this ELISA system offers the opportunity to develop a system in which one serum sample may be tested simultaneously against a variety of different antigens coated to the same carrier. This may be of special interest for SPF screening programs of rodent colonies.

The ELISA proved much more sensitive than the IFA and HIA, since the titers were about 30 and 300 fold higher, respectively. The high sensitivity was reflected also by the detection of the first specific anti-K virus antibodies about 4 and 18 days earlier, respectively. The observed relative late development of HIA antibodies after K virus infection in mice is in agreement with the observations of Greenlee (13), who also showed that specific HIA antibodies were first detected between 15 and 19 days after infection.

The specificity of the ELISA was confirmed by showing that antibody was not detected in sera from the sham-infected animals, SPF mice or in the reference antipolyomavirus serum.

Taken together, the data obtained by the relative sensitivity and specificity of the ELISA clearly show that it may be used for the routine serologic diagnosis of K virus infections in mice.

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References


Figure 2 Relationship between anti-K virus antibody titers in individual mouse serum samples, measured in ELISA, IFA and HIA and shown in a three-dimensional plot.
Footnotes
1The American Type Culture Collection, Rockville, MD, USA.
3ICI, Cheshire, England.
4Microbiological Associates, Bethesda, MD, USA.
5Costar, Cambridge, MA, USA.
6Cappel Laboratories, Cochranville, PA, USA.
7Titertek Multiskan, Flow Laboratories, McLean, VA, USA.