Recent cases in the german poxvirus consulting laboratory

S. ESSBAUER, H. MEYER, O.-R. KAADEN and M. PFEFFER

SUMMARY
Designated as poxvirus consulting laboratory by the Robert-Koch-Institute (Berlin), we provide scientific advice regarding any aspects of poxviruses affecting different animals e.g. cats, elephants, swines, birds, and men. Human smallpox was eradicated in the 1980s, and consequently with diminishing vaccination a generation susceptible for other zoonotic poxviruses grows up. Although the epidemiology of orthopoxvirus infections in Germany remains unclear in the last few years we observed a drastic increase of zoonotic poxvirus infections with three case reports on human «cowpox» infections presented here. In two cases we could only retrospectively trace the source of the orthopoxvirus to cats based on seroconversion. In one case a young cat transmitted the virus to three humans whom all developed clinical pox lesions. Beneath the zoonotic potential of cowpoxviruses (CPXV), these viruses exhibit a broad host range. In the year 2000 two elephants (Elephas maximus) of a German travelling circus revealed a fatal orthopoxvirus infection. The animals exhibited many poxviral lesions and died of this infection. Thus we provide the modified vaccinia virus Ankara (MV A) for vaccination of exotic or expensive animals. Classical virological methods as well as molecular biological techniques including PCR, sequencing and restriction fragment patterns of the newly isolated poxviruses show a very close relationship of the investigated CPXV isolates irrespective of their host species. These findings and our long-term data give evidence of an increase of orthopoxvirus infections in animals and men and underline the importance of further investigations on virus transmission and orthopoxvirus reservoirs.

RÉSUMÉ
Cas récents au laboratoire allemand de référence sur les poxvirus. Par S. ESSBAUER, H. MEYER, O.-R. KAADEN et M. PFEFFER.


KEY-WORDS : poxvirus - cowpox - orthopoxvirus - zoonosis - cats - elephants.


Introduction

In 1980 the eradication of human smallpox was proclaimed. Consequently with diminishing vaccination a susceptible generation without vaccination against poxviruses grows up now. This vaccination also protected against the members of the genus orthopoxvirus (OPV) of the family Poxviridae which do not have humans as their primary host, but harbor zoonotic potential. Recently there are other members of the orthopoxvirus genus, e.g. monkey poxvirus in Africa, or pet hosts, e.g. cowpox virus (CPXV) most frequently in Europe, that include pathogens of human and veterinary importance [9, 20]. Large outbreaks of the monkeypoxvirus in humans have been reported in 1996 to 1998 and it is feared that monkeypox infections inhabit the ecological niche of variola virus and therefore the risk for humans is rising [5, 15].
Cowpox virus has a broad host range and is believed to persist in a reservoir comprising various rodents indigenous to parts of Europe and adjoining Asia, cattle and zoo animals. Very often domestic cats cause intermediate CPXV infected hosts, which may transmit the virus to humans [4, 6, 19].

We are designated as poxvirus consulting laboratory by the Robert-Koch-Institute (Berlin). Every year we get about 500 demands for laboratory confirmation of suspected poxvirus infections. Further, we provide scientific advice regarding any aspects of poxviruses affecting different animals. Table I gives an overview of techniques and their primary application with respect to the poxvirus genera. Routine diagnosis includes electron microscopy, virus isolation and serological and molecular investigations.

Here we present several cases of orthopoxvirus infections of the last two years: CXPV have been isolated from human beings and a cat (*Felis silvestris f. catus*), as well as two elephants (*Elephantus maximus*) whom have been suffering on an OPV infection. Also we report two interesting zoonotic cases were we could show that an CXPV has been transmitted from a cat to men. In one case a kitten transmitted the virus to three human beings by scratching; all patients developed clinical pox lesions.

### Material and methods

#### SAMPLE PREPARATION AND VIRUS DETECTION

Attempts were made to propagate viruses from pustule fluids or scabs. Tissues of patients were homogenized with sea sand in phosphate-buffered saline (PBS) with antibiotics (300 IU/ml penicillin, 300 µg/ml streptomycin). Samples were subjected to ultrasonication for three periods each of 15 seconds. The cell debris was removed by centrifugation for 10 min at 6,000 x g and supernatants were stored at -20°C.

An 10 µl aliquot of the supernatant was allowed to evaporate on a grid. Negative staining was performed with 1 % phosphotungstic acid. The preparations were examined using a Zeiss EM 10 C/R electron microscope.

### Table I

<table>
<thead>
<tr>
<th>Method</th>
<th>Examples of application</th>
<th>Examples of investigations and/or cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron microscopy</td>
<td>Orthopoxviruses (OPV)</td>
<td>OPV of cat, men, elephants (see this paper)</td>
</tr>
<tr>
<td></td>
<td>Parapoxviruses (PPV)</td>
<td>PPV of camel, men, sheep</td>
</tr>
<tr>
<td>Isolation</td>
<td>MA 104 cell line</td>
<td>Cultivation and investigation of new OPV isolates (see this paper)</td>
</tr>
<tr>
<td></td>
<td>Vero cell line</td>
<td>Cultivation and investigation of PPV</td>
</tr>
<tr>
<td></td>
<td>PK 15 cell line</td>
<td>Cultivation and investigation of suipoxviruses e.g. investigation of swinepox virus outbreak in fall 2002</td>
</tr>
<tr>
<td></td>
<td>Chicken embryo fibroblasts</td>
<td>Propagation of modified vaccinia virus Ankara</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cultivation and investigation of avipoxviruses e.g. peregrine falcon isolate in spring 2001, houbara poxvirus from Dubai</td>
</tr>
<tr>
<td></td>
<td>Quarrel cell line (QT)</td>
<td>Cultivation and investigation of avipoxviruses</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>14 kDa PCR</td>
<td>Evidence for OPV infections (see this paper)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e.g. monitoring of molecular epidemiology of OPV, screening of wild rodent populations for OPV</td>
</tr>
<tr>
<td></td>
<td>ATI- PCR</td>
<td>Differentiation of new CPXV isolates (see this paper)</td>
</tr>
<tr>
<td></td>
<td>TK-PCR</td>
<td>Differentiation, characterization of swine poxviruses</td>
</tr>
<tr>
<td>Plaque-reduction neutralization test</td>
<td>Orthopoxviruses</td>
<td>Investigation of OPV antibody titers in human, cats, elephants sera</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e.g. identifying infections retrospectively (see this paper), monitoring CPXV infections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monitoring vaccination of zoo animals with modified vaccinia virus (MVA)</td>
</tr>
<tr>
<td>Enzyme linked immuno-sorbent assay</td>
<td>Competitive OPV ELISA</td>
<td>Investigation of OPV antibody titers in human, cats’, elephants’ sera</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e.g. identifying infections retrospectively, monitoring OPV infections</td>
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<tr>
<td></td>
<td>Competitive PPV ELISA</td>
<td>Investigation of PPV antibody titers in humans, sheepens</td>
</tr>
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<td></td>
<td></td>
<td>e.g. identifying infections retrospectively, monitoring PPV infections</td>
</tr>
</tbody>
</table>

Table I. — Main fields of diagnostic methods and investigations in the German poxvirus consulting laboratory.
Confluent African green monkey kidney MA 104 cells (ATCC No. CRL-2378) were grown in minimal essential medium (MEM) containing 10 % fetal calf serum (FCS). Inoculation (100 µl, 10 µl and 1 µl of supernatant) and propagation of viruses in vitro was performed as previously described [7, 16].

For PCR amplification viral genomic DNA was isolated from 200 µl of infectious tissue culture supernatant or semi-purified skin homogenates using the QIAamp blood® kit referring to the manufacturer’s instructions (Qiagen, Hilden). The DNA was eluted in 100 µl elution buffer provided with the kit.

SEROLOGICAL INVESTIGATIONS

Sera of patients (see table II) were stored at -20°C and inactivated by heating at 56°C for 30 min before use.

Plaque-reduction neutralization test (PRNT)

Plaque-reduction neutralization tests were performed with the vaccinia virus strain Munich 1 (VV strain M1) and MA 104 cell lines as described previously [8]. In brief, sera were diluted in log₂-steps, afterwards the equal volume of 100 plaque-forming units (100 PFU) VV strain M1 was added. After an incubation period for 2 h at 37°C, the mixtures were transferred on MA 104 cells and adsorbed at 37°C for 1 hour. Infected cell cultures were incubated at 37°C and the cells were stained with formalin-crystal violet at 30 h p.i. The PRNT titer was calculated according that serum dilution causing 50 per cent PFU reduction compared with the PFU of virus controls.

Competitive OPV-Enzyme linked immuno-sorbent assay (ELISA)

With the available sera of patients and animals (see table II) an blocking ELISA was performed as described in detail by CZERNY et al. [8]. 96 well microtiter plates were coated with 1 µg/ml VV strain MVA. Sera were titred in log₂-steps and incubated for 60 min at 37°C. Subsequently, an OPV-monoclonal antibody (3D11) was incubated with the test sera. The reaction was detected with peroxidase conjugated anti-mouse IgG (Sigma-Aldrich, Deisenhofen ; 1 : 2000) and 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, Deisenhofen). The optical density was measured at 450 nm (670 nm). A ≥ 50 % reduction of the extinction was regarded as significant.

PRIMERS, PCR AND SEQUENCING

A PCR with primers (14-up : 5'-ATGGACGGAACCT-CTTTTCCC-3' ; 14-low : 5'-TAGCCAGAGATACA-TAGCCGC-3') targeting the vaccinia virus gene of the 14 kDa protein (A27L) was performed. 1 µl of the total DNA was mixed with 0.2 mM dNTPs, 130 ng of each primer, 1 Unit Taq DNA Polymerase (Roche, Mannheim) and 1.5 mM MgCl₂ with Taq buffer provided by the manufacturer in a final volume of 50 µl. The DNA’s were initially denatured at 94°C for 7 min. After five touch-down cycles (94°C, 1 min ; 70°C to 60°C, 1 min ; 72°C, 1 min) the mixture was cycled 40 times (94°C, 1 min ; 60°C, 1 min ; 72°C, 1 min), followed by a final extension for 10 min at 72°C. Amplified DNA fragments (5 µl) were separated by agarose gel (1 %) electrophoresis and purified using the QiagenQuick Kit (Qiagen, Hilden). Both strands of the PCR-fragments were sequenced with the 14-up and 14-low OPV-specific primers (Sequencing service : MediGenomix, Martinsried).

The PCR specific for the gene encoding the acidophilic inclusion protein (A-type inclusion body, ATI) was performed as described by MEYER et al. [17]. In brief, 1 µl of viral DNA was mixed with 20 pmol of sense and anti-sense primer (ATI-up-1: 5'-AATACAAGGAGGACTCT-3' ; ATI-low-1 : 5'-CTTAACTTTTTCTTCTC-3'), 4 mM dNTPs, 3.5 mM MgCl₂ and 0.5 U Taq-Polymerase (Gibco BRL, Eggenstein). The PCR mixture (50 µl) was denatured at 94°C for 7 min. After five touch-down cycles (94°C, 1 min ; 60°C to 50°C, 1 min ; 72°C, 2.5 min) the mixture was cycled 40 times (94°C, 1 min ; 50°C, 1 min ; 72°C, 2.5 min), followed by a final extension for 10 min at 72°C. The ATI-specific PCR products of the CPXV isolates and the VV strain M1 were differentiated by enzyme digestion with 20 U XbaI (New England Biolabs, Frankfurt). Aliquots of the PCR products as well as the digested DNA fragments were separated by agarose gel (2 %) electrophoresis.

Table II. — Diagnostic and laboratory findings of German orthopoxvirus cases investigated in this study.
For both PCRs vaccinia virus strain M1 DNA served as positive control, aqua dest. was used as negative control. All amplifications were performed in a Cycler PE 2400 (Perkin Elmer, Weiterstadt).

Results

We investigated five different cases of orthopoxvirus infection of animals and men. Table II gives an overview of the cases and the investigations performed during this study.

CASE 1: POXVIRUS INFECTION OF A MAN

Case

During a camping trip in early summer 2001 a 21-year-old man obtained a small papule, like an insect bite, medial on the left upper eye-lid. The diameter of the papule increased to about 2x2 cm and after several days it incrusted beginning from the center. After one week the man was presented to hospital and stayed four days. Eleven days after the appearance of the first papule the patient developed fever and a tender local, preauricular, cercical and submental lymphadenopathy. On the eye-lid an periorbital edema appeared. The patient was treated with Augmentan® in order to prevent bacterial super infections. The histology and the complete blood count were without significant changes. The patient had contact to a cat living on a farm. The cat had influenza-like symptoms several weeks before the man got infected.

Results

Virological methods for the diagnosis of poxvirus infection included the direct visualization of viruses by electron microscopy and by multiplication of the isolated virus in cell cultures (e.g. MA 104). Using the negative staining technique, brick-shaped particles with a diameter of about 200 x 300 nm, typical for orthopoxviruses, were detected in skin biopsies (fig. 1a). A cytopathogenic effect developed within 2-5 days in the monkey kidney cell line, producing small plaques (see fig. 1b, c). Due to the morphology under the electron microscope, diagnosis of an orthopoxvirus infection was confirmed. At the day 1 of the onset of symptoms and three weeks after the infection the serum of the patient was investigated for anti-OPV specific antibodies. Serum samples of day 1 and 21 days exhibited a titer of 1:128 in a PRNT. In contrast, attempts to identify virus particles in skin biopsies of the cats inner and outer lip by electron microscopy or by attempts to cultivate the virus in MA 104 cells failed. However, we could retrospectively show the feline infection as the cats serum revealed an titer of 1:1024 in an OPV-PRNT.

CASE 2: POXVIRUS INFECTION OF A GIRL AND A CAT

Case

A 10-year-old girl presented to hospital in June 2001 with severe erythematous eruption behind the ear, around anus and vagina, on the hands, the feet and the back. As a streptococcal superinfection appeared, an antibiosis was performed. The girl stayed in hospital for almost three weeks. The lesions were treated with substances locally drying up. The infection healed slowly (about 8 weeks), leaving no scars. The patient had close contact to her cat. The cat was reported to have had crusty lesions in the face four weeks before the girl got afflicted.

Results

Histopathological investigation of a skin biopsy of the girl revealed CPXV-like inclusion bodies of affected cells. Electron microscopy and isolation of virus in cell culture proved the suspected CPXV infection. A sero-conversion after 10 days could be demonstrated using OPV-PRNT (titer 1:1024). In contrast, attempts to identify virus particles in skin biopsies of the cats inner and outer lip by electron microscopy or by attempts to cultivate the virus in MA 104 cells failed. However, we could retrospectively show the feline infection as the cats serum revealed an titer of 1:1024 in an OPV-PRNT.

CASE 3: POXVIRUS INFECTION OF THREE HUMAN BEINGS AND A CAT

Case

A 20-year-old woman was injured by a neighbors kitty while playing with it. After two days the woman developed two lesions with a diameter of 2 cm on the forearm. The lesions later became conflaunting. After two weeks further crusts with a diameter of 1 cm were obvious on the head and on the groin. 16 days later a lymphangitis developed from the wound that could be treated with antibiotics (oxacillin, erythromycin) and antiseptics (betaisadona). However, the local lymph nodules showed a persistent swelling and several nodular skin alterations were obvious. As the woman was injured by a 6-week-old kitty cat that was separated from its mother two weeks before and whose mother was frequently hunting mice, we tried to get samples from the kitty that hit her. Interestingly, the kitty exhibited crusts on the head and ears. Meanwhile, father (54 years) and son (14 years) of the cat’s household also exhibited 2-3 pocks on their arms and foreleg. However, the lesions of the son were more severe at that of the father. No secondary pox lesions were noted. All patients’ lesions healed with leaving scars.

Results

Histopathological investigations of skin biopsies of the woman revealed eosinophilic inclusion bodies in the epidermis. In a crust biopsy of the woman orthopoxvirus-like particles were detected by negative staining in the electron microscope. A cytopathogenic agent induced plaques 24 hours p.i. at 37°C in MA 104 monkey kidney cells. A serological investigation of the patients serum (after 16 days and 41 days) revealed each a OPV-antibody-titer of 1:1024. In contrast, attempts to identify virus particles in skin biopsies of the cats inner and outer lip by electron microscopy or by attempts to cultivate the virus in MA 104 cells failed. However, we could retrospectively show the feline infection as the cats serum revealed an titer of 1:1024 in an OPV-PRNT.

FIG. 1. — (A) Electron micrograph of negative-stained cowpox viruses. Virions reveal the typically brick-shaped morphology of poxviruses. Magnification : 64,000 x (B, C) Micrograph of cell cultures infected with CPXV isolate from case 1. (B) MA 104 cells, mock-infected (control). (C) The infected MA 104 cells revealed cytopathogenic effects, e.g. plaque-formation after 24 h p.i. (B, C : Magnification : 125 x).

FIG. 2. — Patients exhibiting pocks. (A) Poxviral lesions on the ear of a kitty (case 4). (B) One elephant (case 5) exhibited pocks on his upper eye lid and (C) several lesions above his carpal joint.

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Samples of the son that also exhibited pocks on his forearm have not been investigated.

In summary, we could show CPXV infection of a cat and of three clinically affected people, the cats owners and a neighbor.

CASE 4 : POXVIRUS INFECTION OF A KITTEN

Case

In autumn 2001 a 11-weeks-old, male kitty that exhibited multiple round, red papules on the face, head (fig. 2a) and right hind foot was presented. The claws of this foot were purulent. The kitty also had a rhinitis which became more severe after five days. The kitty was since four days with a new owner and originated from a farm in Thuringia. Further incrustations were obvious e.g. in the oral cavity. The kitty was euthanized.

Results


CASE 5 : POXVIRUS INFECTION OF TWO ELEPHANTS (ELEPHAS MAXIMUS)

Case

In September 2000 an outbreak of a poxvirus infection appeared in two elephants of a German traveling circus. The animals exhibited several pox viral lesions. One elephant had a large pock on his palperal fissure and a red papule over the other eye (fig. 2b) The disease became more severe and generalized. The animals showed multiple ulcerative, weeping lesions on their knees, on their forefoot, especially slightly proximal of the carpal joint (fig. 2c). Further pocks appeared on inner and outer mucosa of the snout and mouth. Treatment with antibiotics and trials to save the animals by weight relief, as the pocks were localized on the carpial joint and the feet, failed. The elephants died one month after the fatal OPV infection.

Results

Electron microscopy of crusty biopsies of the elephants revealed orthopoxviruses. The orthopoxvirus infection was further proved by PCR analyses as described in the next paragraph. As the scab samples contained multiple bacteria and fungi the material could not be used for virus isolation in cell cultures. Experiments to demonstrate an acute viremia by inoculation of the elephants sera in MA 104 cells failed.

MOLECULAR COMPARISON OF THE ISOLATES OF THE FIVE CASES

Virus material either obtained from tissues (case 5) or obtained after cultivation in MA104 cells (case 1 - 4) was further processed for DNA-isolation and OPV-specific polymerase chain reactions.

The PCR with primers targeting the 14 kDa protein gene of OPV resulted in 280 bp-sized DNA fragments for the all investigated OPV isolates of human beings, cats and elephants (data not shown). Since the PCR yielded an 280 bp amplicon with the same sequence for all OPV-species we applied a second PCR procedure which achieves an identification and differentiation of OPV-species by the use of consensus primers targeting sequences of the ATI-gene of cowpox virus. After DNA isolation, we could amplify ATI-gene PCR products of approximately 1600 bp in size. After XbaI cleavage of the amplicons derived from all cases reported here, DNA fragments of about 645, ~500 (515), 346, 100 and ~100 (67) bp were obtained (fig. 3, lane 2-8). This pattern corresponded exactly to the one found in German CPXV isolates, and differs from a XbaI digest of vaccinia virus strain derived ATI-PCR amplicon (900, 320, 220, 100 and 70 bp ; fig. 3, lane 9). Hence, CPXV isolates either from men, cats or elephants that can be distinguished from the vaccinia virus clearly belong to the common CPXV-type found in German.

Discussion

In this study we present data of five cases of OPV infections. Two have retrospectively been shown to be zoonotic transmitted, one up to three humans. In general the CPXV serologically crossreacts with other OPV in PRNT and OPV-ELISA. Several studies of different CPXV strains and isolates demonstrated that CPXV consists of closely related variants with different characteristics [2, 17]. Here, electron microscopy, cell culture, PRNT and/or competitive ELISA, and two different OPV-specific PCR’s confirmed the presence of CPXV in all investigated five cases irrespective of the host species.

Comparison of 14 kDa Protein (A27L) PCR sequences revealed no differences. The A27L fusion protein of vaccinia virus is needed for the formation of the intracellular enveloped particles [24]. So far, more than 100 OPV strains and isolates have been successfully amplified with this PCR (MEYER H., unpublished data). Due to specific point mutations sequencing of the amplicons allows clear differentiation of viral species [6, 22, MEYER H., unpublished data].

Comparison of restriction fragment pattern of ATI-PCR products revealed no differences for the new CXPV, however these differ clearly from VV strain M1. It has been shown that exclusively cowpox, ectromelia and racoonpox virus produce visible ATI-proteins [17]. By comparing the ATI-PCR results with these of former studies [18], it can be concluded that the CPXV isolates of elephants, described in this study, and all other investigated viruses belong to a group of isolates described from man but differ from elephant poxviruses EP-1, EP-2, EP-3 and EP-4. These results indicate again the close relationship of the isolates irrespective of the host species.

In the kitten of case 2 we could retrospectively demonstrate an CPXV infection using serological methods. However, we could not prove poxviruses by electron microscopy or by isolation of virus in cell culture. In order to investigate the presence of CPXV we also tried to amplify DNA from the kitten tissues. However, both the 14 kDa- and the ATI-PCR resulted
in no amplicon indicating the absence of the poxvirus which already disappeared.

The first CPXV infection of a domestic cat was reported in Britain in 1977 [25]. Transmission of orthopoxviruses from cats to man results from intense direct contact with the animal or from indirect contact with pox lesions of affected animals in skin abrasion or open wounds [10]. CPXV infections are rare today the virus can sometimes induces a relatively severe zoonosis. The virus has been transmitted from both zoo animals and domestic cats to humans, e.g. owners [1]. A fatal, deadly case of a CPXV infection of an immunosuppressed person was reported in 1991 [7].

We describe orthopoxvirus infections of circus elephants. Since 1963 poxvirus infections of elephants are repeatedly reported from European countries including Germany. It has been shown that these viruses are antigenetically and genetically closely related to CPXV [2, 19, 23, 26]. Both investigated elephants had not been vaccinated with the modified vaccinia Ankara (MVA) that is offered by our laboratory. Our institution has 15 years of experience in providing the MVA for vaccination of exotic or exotic animals such as elephants, tapisrs and rhinoceroses. The strain MVA is highly attenuated and differs pheno- and genotypic (deletions) from the original Ankara virus. The virus has a restricted host spectrum, reduced virulence and contagiosity in man and animal [4, 13].

What are the reservoirs for CPXV? OPV specific antibodiess and virus sequences have been detected in rodents from several European countries [6, 11, 12, 14]. It was strongly suggest that several rodent species and for screws are responsible for maintaining CPXV in nature.

In summary the German poxvirus consulting laboratory long-term data provide evidence of an increase of OPV infections in animals and men. It is important to focus on CPXV as this zoonosis is rising and the risk for people becoming infected due to close contact to animals e.g. cats. Our long term experience, and the presented recent cases indicate the importance of further investigations on virus transmission and OPV reservoirs.

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References