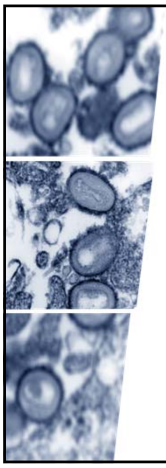

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Zoonotic poxvirus infections in Europe

Andreas Nitsche

Robert Koch Institute
Centre for Biological Threats and Special Pathogens
Highly Pathogenic Viruses
German Reference Laboratory for Poxviruses
Berlin, Germany

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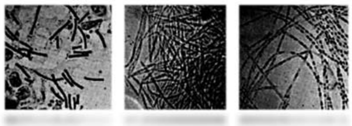
Robert Koch

- 1905 Nobel Prize
  - "…investigations and discoveries in relation to tuberculosis"
- 1876 Isolation of *Bacillus anthracis*

Original micrographs by Robert Koch



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The Robert Koch Institute - today

Today the Robert Koch Institute is a Federal Institute within the portfolio of the German Federal Ministry of Health

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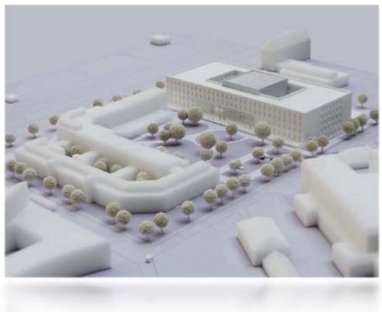
The Robert Koch Institute - 2015



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Centre for Biological Threats and Special Pathogens (ZBS)

- ZBS 1: Highly Pathogenic Viruses
- ZBS 2: Highly Pathogenic Bacteria
- ZBS 3: Microbial Toxins
- ZBS 4: Advanced Light and Electron Microscopy
- ZBS 5: BSL4 Lab
- ZBS 6: Proteomics and Spectroscopy
- IBBS: Federal Information Centre for Biological Threats and Special Pathogens

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ZBS 1 tasks

Viruses of risk group 3/4 – viruses with BT-potential – viruses for differential diagnosis
imported Viruses – new and emerging viruses – modified viruses

Diagnostic	Pathogenesis	Therapy/prophylaxis
Nucleic acid Antigen Serology Reference methods SOPs Reference material	Virus-host-interactions *host-range*-effects Animal models	Antiviral substances Antibodies/Immunotherapy Aptamers

- Dr. Heinz Ellerbrok
- Dr. Livia Schünadel
- Prof. Dr. Matthias Niedrig

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Bioterrorism

A terrorist act involving the use of harmful agents and products of biological origin, as disease-producing microorganisms or toxins

Criteria CDC classification	Popular candidates
Dissemination	Anthrax
Transmission	Botulism
Mortality	Plague
Public health impact	**Smallpox**
Preparedness	Tularemia
	Filoviruses
	Arenaviruses
	Viral encephalitis viruses
	Hantavirus
	Nipah virus

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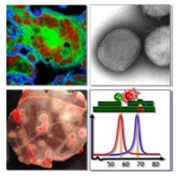
German Reference Lab for Poxviruses

Dr. Andreas Nitsche, Dr. Livia Schünadel

- since 2005

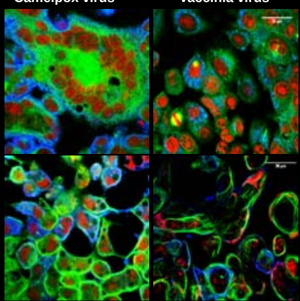
- Diagnostic of (human-)pathogenic poxviruses
- Since 2005 ~3500 samples from >200 cases
- Trainings for Public Health institutions
- "Accreditation" DIN ISO 17025 15189, May 2010



Serology and EM can differentiate genera but not species !!!

Immunofluorescence assay

Camelpox virus Vaccinia virus

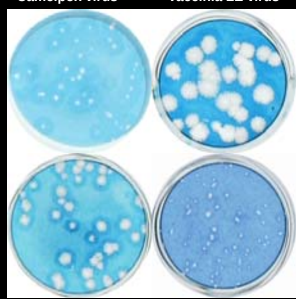


Cowpox virus Ectromelia virus

Daniel Stern

Plaquetest

Camelpox virus Vaccinia LE virus

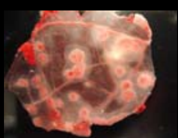


Cowpox virus Ectromelia virus

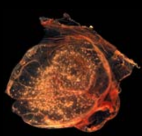
Daniel Stern

Chorioallantoismembran (CAM)

Vaccinia virus 41°C



Cowpox virus 40°C



Variola virus 37.5-38.5°C

ceiling temperature

RKI, WHO

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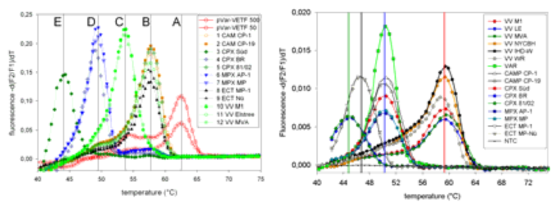
Real-time PCR



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The poxvirus family

Genera pathogenic to humans

Family	Subfamily	Genus	Species
Poxviridae	Chordopoxvirinae	Avipoxvirus	
		Capripoxvirus	
		Cervidpoxvirus	
		Crocodylidpoxvirus	
		Leporipoxvirus	
		Molluscipoxvirus	
		Orthopoxvirus	
		Parapoxvirus	
		Suipoxvirus	
		Yatapoxvirus	
	Entomopoxvirinae	A	
		B	
		C	

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The poxvirus family

Species pathogenic to humans

...in Europe

Avipoxvirus
Capripoxvirus
Cervidpoxvirus
Crocodylidpoxvirus
Leporipoxvirus
Human-specific → Molluscipoxvirus
Orthopoxvirus ← Vaccinia virus, Variola virus, Monkeypox virus, Cowpox virus, Camelpox virus, Ectromelia virus, Taterapox
Bovine Papular Stomatitis Virus, ORF, Pseudocowpox virus → Parapoxvirus
Suipoxvirus
Yatapoxvirus
Tanapox virus
Yaba Monkey Tumor virus

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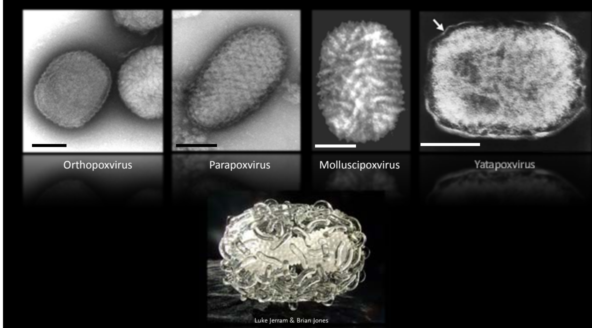
How do they look like?



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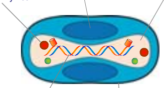
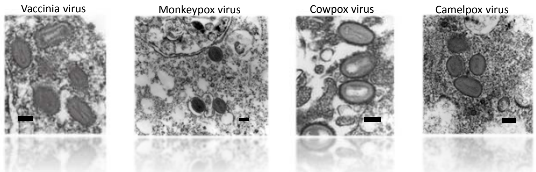
Orthopoxviruses

- particle size: 200 x 250 x 300 nm
- genome: dsDNA
  - 190.000 to 230.000 bp
  - ~ 200–250 genes
- replication in cytoplasm





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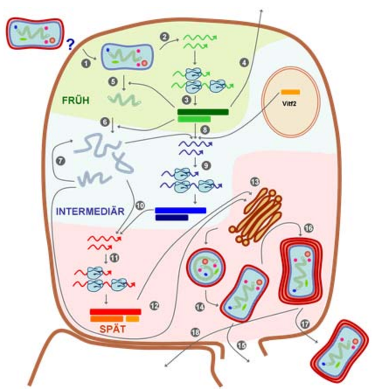
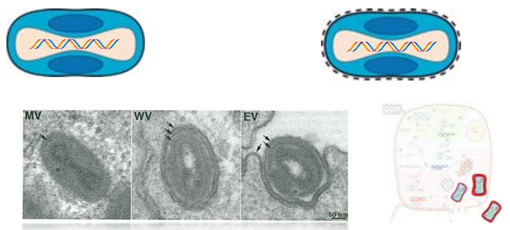
Replication



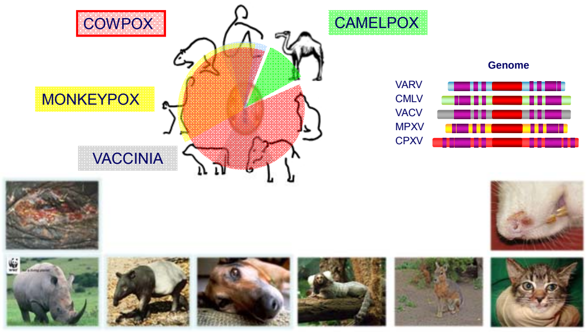
Two particle form

- *Intracellular Mature Virus*
  - IMV MV
  - cell lysis
  - cell-to-cell infection
  - A27L, L1R, D8L, H3L, F9L
- *Extracellular Enveloped Virus*
  - EEV EV
  - distribution in the organism
  - A33R, B5R, F13L

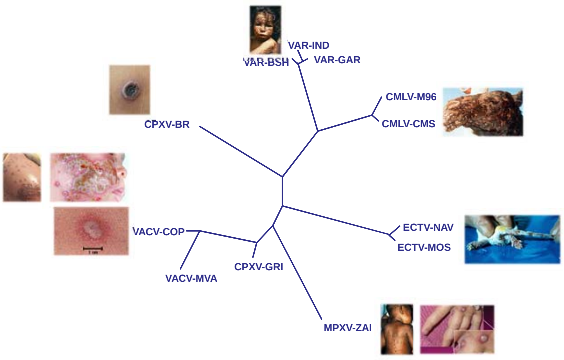


Fields Virology

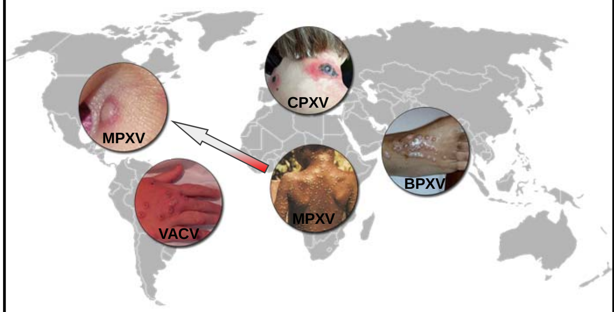
OPV host-range



Orthopoxvirus Phylogeny



Zoonotic OPV infections

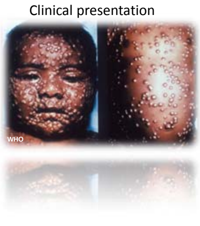


Sources: CPXV: RKI, MPXV: WHO/CDC, VACV: CDC, BPXV: CDC

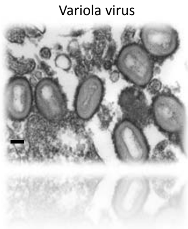
Smallpox

- Variola virus
- Mortality 20-90%
- Approximately 300 million deaths in 20th century
- Smallpox declared eradicated in 1980

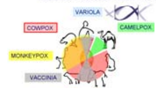
Clinical presentation



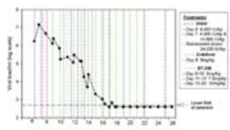
Variola virus



Vaccinia Virus



- Prototype and Vaccine
- Naturally occuring in South America
  - Vaccine strain circulating for decades?
  - Infections from cattle
- Transmission following vaccination
  - ♂ 28 month
  - Atopic dermatitis
  - Eczema vaccinatum
  - father: US-soldier, VACV
  - Frist application of ST-246



Trindade et al., Vora et al.

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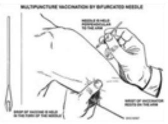
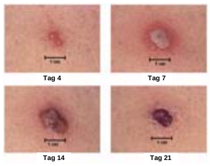
Vaccines

- Vaccinia virus
  - Origin unknown
  - 1939 Vaccinia virus
- Live vaccines
  - 1. Generation: Anzucht in Kälbern, CAM
  - 2. Generation: Anzucht in Zellkultur
- Eradication
  - New York City Board of Health
  - Lister/Elstree
  - Tian-Tan
  - 1950 *freeze-dried vaccines*
- *take* = successful vaccination





CDC

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Eradication

- Vaccination law (Germany 1871)
- WHO-vaccination campaign since 1967
- Last naturally occuring smallpox cases
  - Variola major: Rahima Bahu in Bangladesh 16.10.1975
  - Variola minor: Ali Maov Maalin in Somalia 26.10.1977

WHO: Global Commission for Certification of Smallpox Eradication. December 9th 1979

WHO

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What made eradication possible?

- Infection ALWAYS leads to disease
- No latent or persistent infections known
- No animal reservoir
- High stability of antigens
- Vaccine rise long-lasting immunity
- Vaccine production simple, cheap, stable
  - …in times before molecular biology
- Huge commitment of the *smallpox eradication unit*

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The milestone for vaccinations in 1796!

- What was the virus Jenner used?
- No documented cowpox in cattle since 30 years in Germany/Europe

Is cowpox misnamed?

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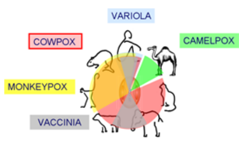
Cowpoxvirus pecularities

- Increasing number of infections in Europe!
- Largest Genome of all OPV, +30kb
- Most complete number of genes
- Heterogeneous population
- Co-circulation in nature
- Various species infected
- Virus-host combination essential for pathogenesis!



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Human cowpox disease

- Incubation period
  - 1–2 weeks
- Symptoms
  - Locally limited self-limiting lesions
  - Exanthema
  - Papule → vesicle → Pustule
  - 3–5 weeks formation of scars
  - Lymphadenitis in regional lymph nodes
  - Generalized exanthema, systemic lethal disease in immunosuppressed patients

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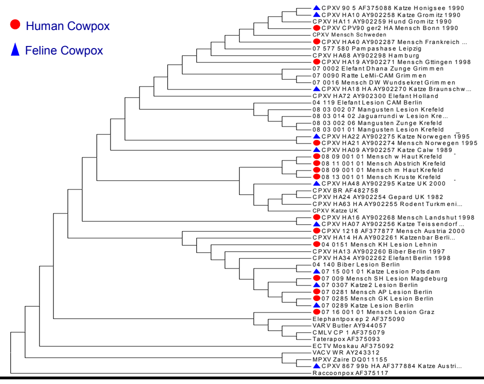
German CPXV are diverse !



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Cowpoxvirus transmission

- No human to human transmission described
- Infection through direct contact to virus containing material
- Common cat-scratches
- Where do cats get infected ?

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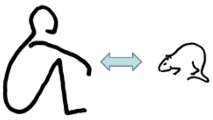
An unusual first case

- 02/08
- Female person
- Contact to rats kept as pets
- Non-vaccinated against OPV

- CPX virus HA-gene divergent from all other strains known so far



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At the same time.......

- 02/08
- Banded mongooses in a zoo
- Same region of Germany

- CPX virus HA-gene divergent from the 1st rat strain and all other strains known so far

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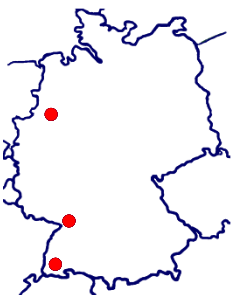
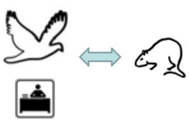
In the 15 following month.......



- Two females
  - One feeding falcons
  - One working in the office a reptile zoo
- Long distances between contact sites

- CPX virus strain identical to strain from the mongooses



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Rats used as animal food

zoo 02/08

reptile Zoo 09/08

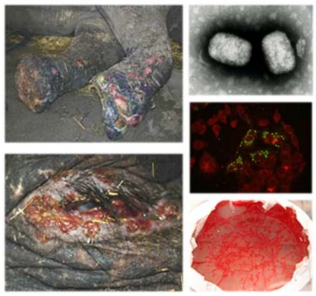
falconry 05/09

CPXV feeder rats ≠ pet rats ≠ common rats
- 2 Human infections (>1 year)
  - w and w/o direct contact
- 14/14 Banded mongooses, 1 Jaguarundi
- ~ 60% of feeder rats

A diseased circus elephant

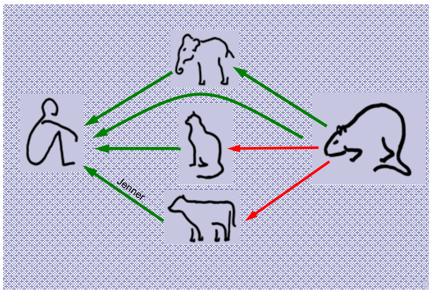
- 02/07
- 50 years old
- Non-vaccinated against OPV

Transmission by common wild rats



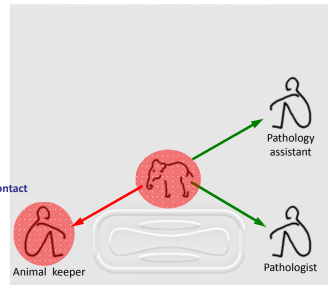
CPXV common rats
≠ pet rats ≠ feeder rats
- 1 Human infections
- ¾ rats PCR positive
- 4/4 seropositive

CPXV Transmission

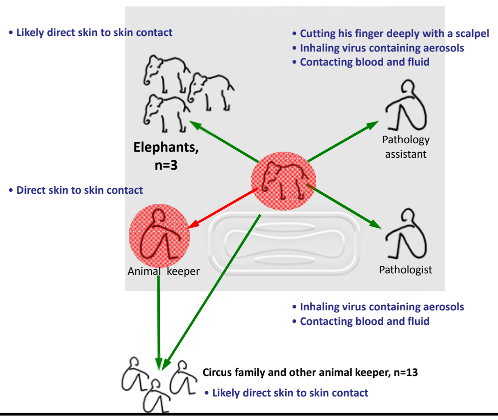


Exposure

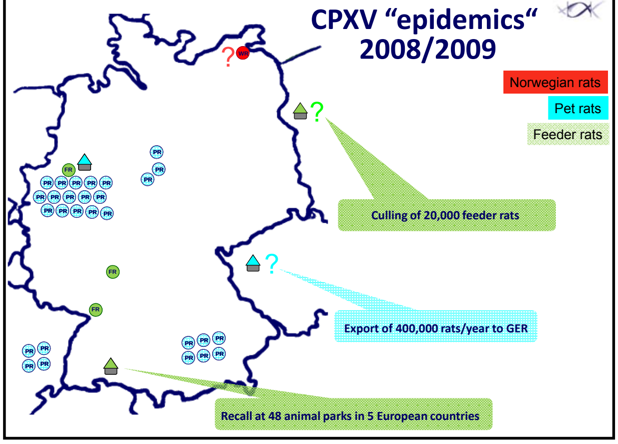
- Direct skin to skin contact



	IgG	IgM	NT
Day 7	1:20	1:40	<1:5
Day 28	1:10240	1:2560	1:80



CPXV “epidemics“ 2008/2009



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Increase of (documented) cowpox

- Halt of vaccinations
  - Waning immunity
- Increased awareness of physicians
- Ecological changes?
  - warm winters favour rodent reproduction ?
- Popularity of rats kept as pets

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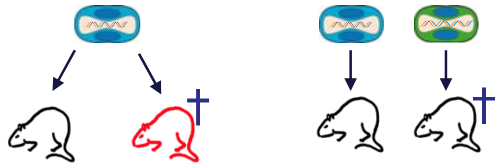
Rats as infection model for CPXV

- Applied research
  - Transmission routes
- Basic Research
  - Virus-specific factors for host/tissue tropism
  - Comparative transcriptomics (host and virus)



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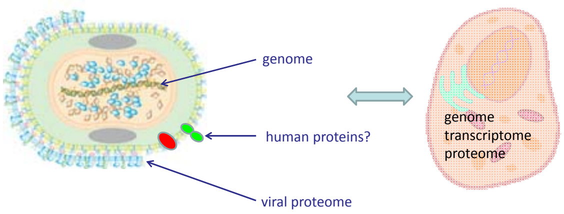
Factors that determine CPX virus-variant/host-species interactions ?



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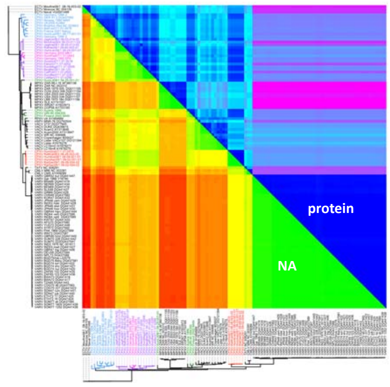
Genome Comparison



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Genetic distances

	BR	HumKre08	RatKre08	EleGri07	MonKre08	RatHei09	HumLan08
BR	100	96.6	96.6	96.9	96.8	96.8	96.8
HumKre08		100	100	97.9	97.9	97.9	97.9
RatKre08			100	97.9	97.9	97.9	97.9
EleGri07				100	98.8	98.8	98.8
MonKre09					100	100	100
RatHei09						100	100
HumLan09							100

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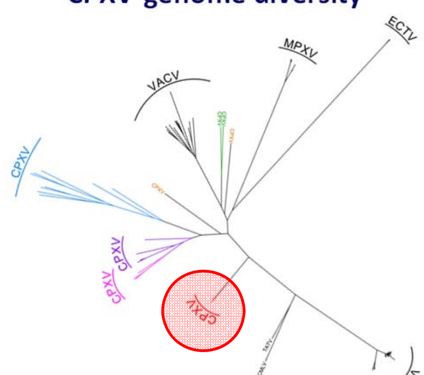
CPXV-genome diversity



- Cowpox-like viruses !!!

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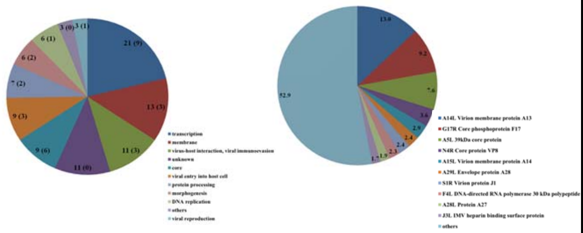
Comparative proteome analysis



nLC/MS/MS-based identification of the conserved proteome of the 3 CPXV strains Brighton Red, CPXV_Hei and CPXV_Kre.

- GO-terms of viral proteins according to frequency in the virion
- Molar percentage of viral proteins in the virions

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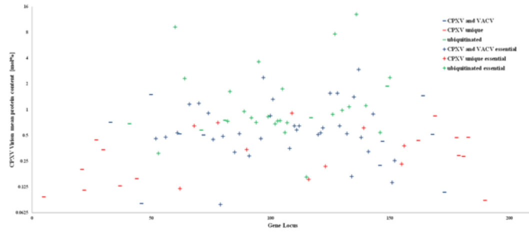
Genome location of virion proteins



- Proteins identified in VACV and CPXV virions are shown in blue
- Proteins specific for CPXV in red

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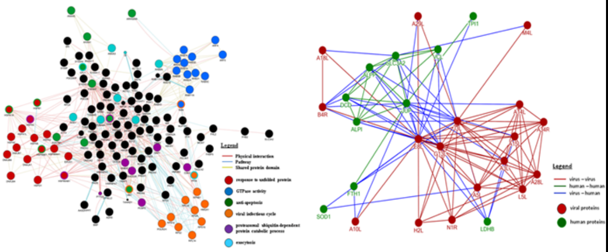
Human proteins in virions



Over-represented GO-terms of human proteins associated with the virion.
Using a BlueNative PAGE – nLC-MS/MS – clustering approach the interactions of CPXV envelope proteins and cell membrane were anaysed globally. Viral proteins, cellular proteins. Complexes of viral plus cellular proteins are connected with a blue line.

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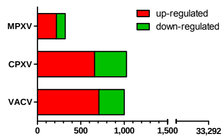
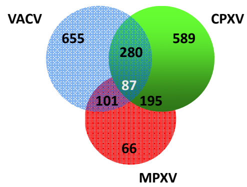
Virus-host interaction: transcriptome

- Microarray analysis
- Comparison CPXV BR-VACV IHD-W-MPXV MSF-6



No. of host mRNAs regulated by infection



Differential regulation of host cell genes

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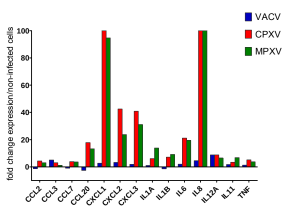
Virus-host interaction: transcriptome

- Gene Enrichment Analysis
- Differential regulation of genes annotated to GO term
  - "Inflammatory response"
- CPXV and MPXV specific up-regulation of several cytokine genes



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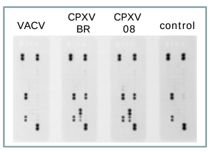
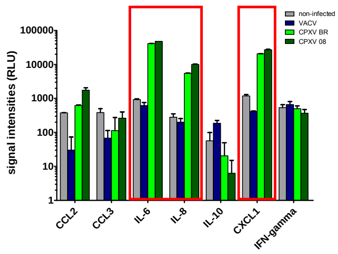
Cytokine secretion

- Verification on the protein level via a human Cytokine Array
- Strong secretion of CXCL1, IL-6 and IL-8 into the supernatant of CPXV, but not VACV infected cells
- Similar effect of two less related CPXV strains: no strain dependency



Cytokine levels in the supernatant of infected or non-infected HeLa cells, 6 h p.I.



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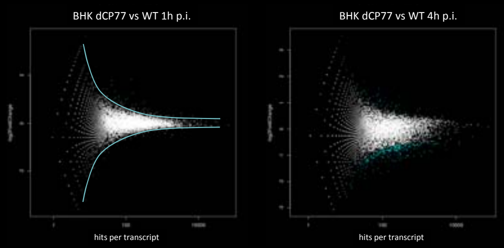
NGS transcriptome analysis

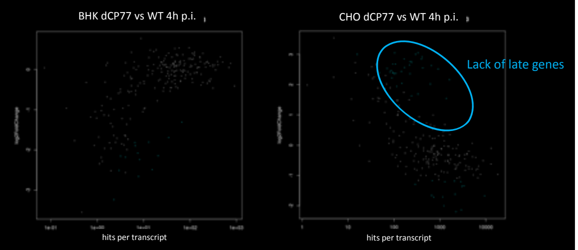
-cellular transcriptome-



- Illumina HiScan SQ
- TruSeq RNA Sample preparation Kit
- FlowCell SR Version 3, SBS Kit v3 100 bases
- 30 million reads per sample

NGS transcriptome analysis

-viral transcriptome-



- Illumina HiScan SQ
- TruSeq RNA Sample preparation Kit
- FlowCell SR Version 3, SBS Kit v3 100 bases
- 30 million reads per sample

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CPXV variability

- Several differences on genome level identified
  - Analysis of individual genes/proteins required
- CPXV strains incorporate different human proteins
- Interacting pathways of the host cell identified
- crmA up-regulation in rat-pathogenic strains (data not shown)
- Investigation of host-species reveals CPXV-specific cytokine regulation
- Cytokine modulation more similar to MPXV than to VACV

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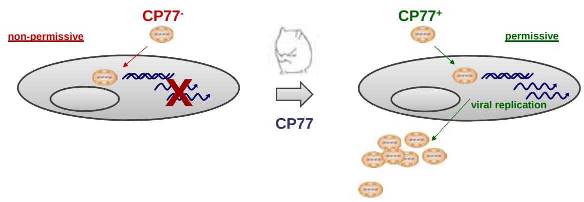
Investigation of individual proteins



- Tropism dependent on repertoire of host range genes
- CP77 permits CPXV replication in CHO cells
- Overcomes replication block at stage of intermediate translation
- BAC mutagenesis to delete CP77

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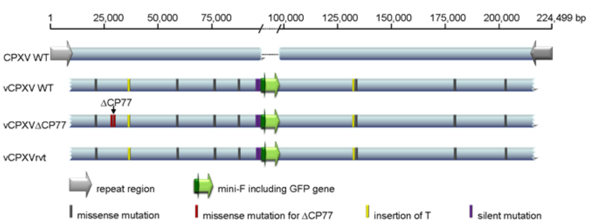
Comparison of whole genome sequences



- 92% of genomes aligned
- No mutations occurred during BAC mutagenesis, reconstitution and propagation of virus

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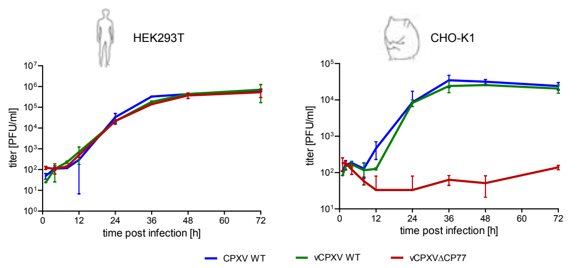
Comparative *in vitro* growth kinetics



- Replication of WT and ΔCP77 comparable in HEK293T
- Replication of ΔCP77 attenuated in CHO

infection with MOI 0.1

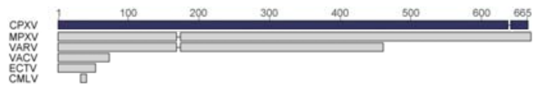
Comparative analysis of late protein expression

- Lack of CP77 results in block of protein expression in CHO cells

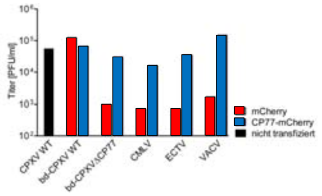
infection with MOI 10

CP77-overexpression

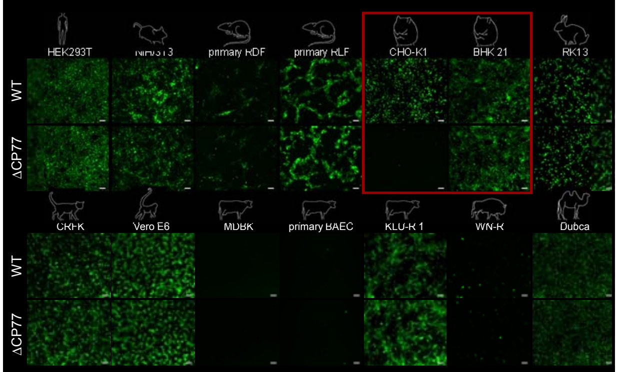
- CP77 protein conservation among OPV



- CP77 restores replication in CHO cells

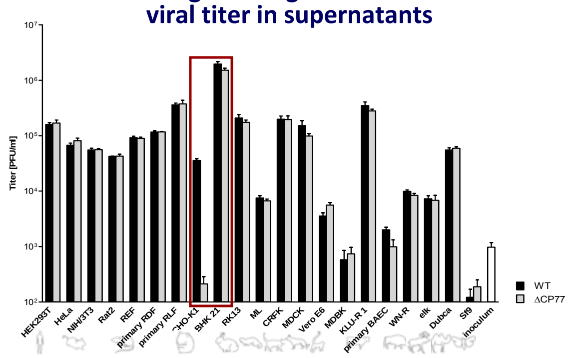


Host range of vCPXVΔCP77



infection with 125,000 PFU, 24h p.i. scale bar = 100µm

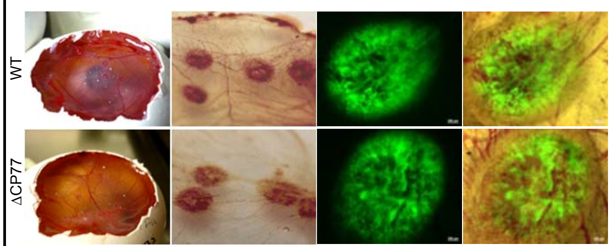
Host range testing of vCPXVΔCP77: viral titer in supernatants



- Comparable replication of WT and ΔCP77 in cells of various species

infection with 125,000 PFU, plaque titration testing 48h p.i.

host-range testing of vCPXVΔCP77: pock morphology on CAM



- Comparable pock morphology of WT and ΔCP77

infection with 200 PFU, 72h p.i.

CP77 – a host range protein ?

- Host range effect only in CHO cells
- No host range effect in further hamster cells (BHK)
- Does CP77 display other functions than host range ?
- What is the cellular binding partner of CP77?

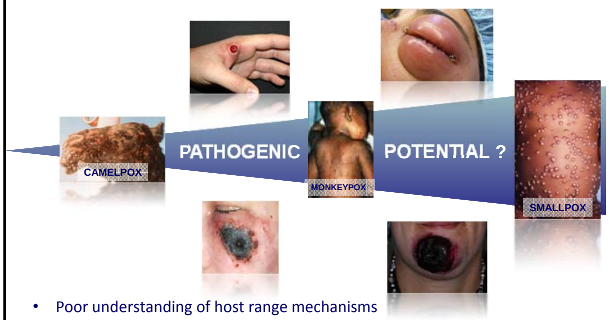
Summary I: General conclusions

- Infections with cowpoxviruses are diagnosed increasingly in Germany/Europe
- Cowpoxviruses have a broad host-range
- 50% of genome encode immunomodulatory factors
- Pathogenic potential depends on specific host/virus combination
- *in vitro* models used to identify relevant factors for interaction
- Appropriate *in vivo* models required

Summary II: Specific conclusions

- Cowpoxviruses are genetically heterogeneous with a new clade closely related to VARV, TATV and CMLV
- Different isolates display varying proteome composition of the virion
- Various cellular pathways identified
- Cowpoxvirus mediated cytokine regulation is more similar to monkeypoxvirus than to vaccinia virus
- Host-range functions are likely not always attributed to one single protein

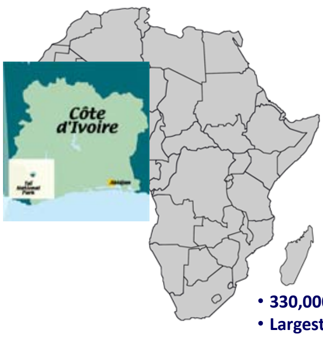
Why to work on poxviruses ?



- Poor understanding of host range mechanisms
- Nearly no understanding of pathogenetic potential
- Beside variola other (new) poxviruses circulating in nature
- Worldwide distribution

Is Monkeypox named correctly?

Taï National Park



- 330,000 hectare
- Largest primary forest in West Africa
- Several endangered species
- UNESCO World Heritage list

Background project

- Division: Epidemiology of Highly Pathogenic Microorganisms Fabian Leendertz
- Zoonosis of relevance for human health originating from non-human primates
- Assessment of the risk of emergence of new zoonotic diseases
- Focus on human populations with exposition to blood/organs of "bush meat"
- Long-term program to monitor death in wildlife

The case

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- Infant mangabey found dead in the TNP in March 2012
  - Died recently, presence of blowfly eggs, absence of maggots
  - No apparent injuries, predators unlikely although leopard around
- Multiple skin lesions typical of OPV infection
  - Dark red crusts 5–7 mm, partly confluent, disseminated over the body
  - Extremities mainly affected; fewer lesions on the belly, none on the back

- No photographs of the diseased mangabey available !!!

Pathology

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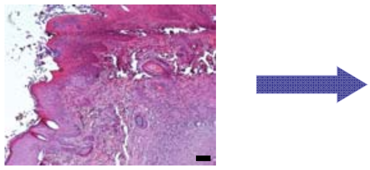
- Full necropsy under high-level safety measures
- Samples of all organs and blood were collected and preserved
- Histology of the skin suggested poxvirus infection
  - eosinophilic inclusion bodies
  - transition of hyperplastic epithelium to severe ulcerative and necrotizing skin alterations
- Severe bacterial secondary infection of the ulcers was observed



New Poxvirus ?
Monkeypox ?

Monkeypox disease

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- First discovery in lab monkeys 1958
- First human case in DRC 1970
- Similar to smallpox but less severe
- Transmission via infected (clinical) material
- Human-to-human transmission possible
  - Longest transmission chain 4 persons

- CFR 1-10%
  - children <16 years most affected

Monkeypoxvirus

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- Typical orthopoxvirus: Size, shape, genome
- Two genetic clades: Congo Basin and West African
- Natural hosts
  - Rodents, squirrels (isolates)
  - non-human primates ? (sero-positive)
- Accidental host: humans (isolates)

No report on MPXV isolates from wild-living monkeys

Molecular diagnostics

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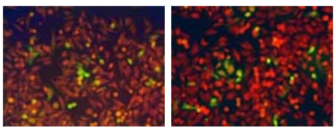
- Real-time PCR for OPV

Tissue	Spleen	Lung	Kidney	Skin	Liver	Heart	Intestine	Muscle	Thymus	Throat swab	Lymph node
CT OPV rpo18	32	34.3	28.2	16.9	30.3	32.2	32.2	n.d.	15.8	21.5	24.1
CT c-myc	22.1	27.8	18.9	26.9	20	25	31.1	n.d.	19.7	28.4	20.2
ΔCT (c-myc– rpo18)	–9.9	–6.5	–9.3*	9.9	–10.3*	–7.2	0.1	n.d.	3.9*	6.9*	–3.9*

* 5 µl DNA used instead of 1 µl

- HA ORF Sequencing: MPXV
  - 99% identical to Liberia_1970_184 acc# DQ011156.1, human
- Serology IFA



IgM 1:80 IgG 1:320

Genome analysis

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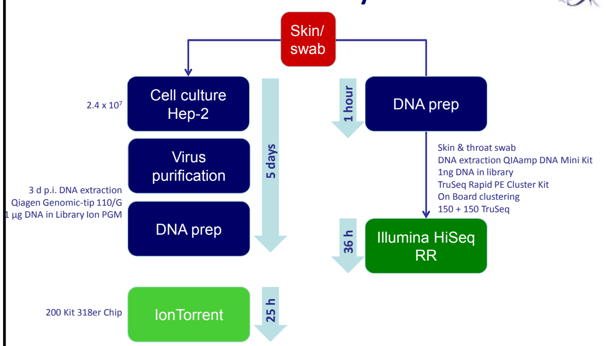

Bioinformatics

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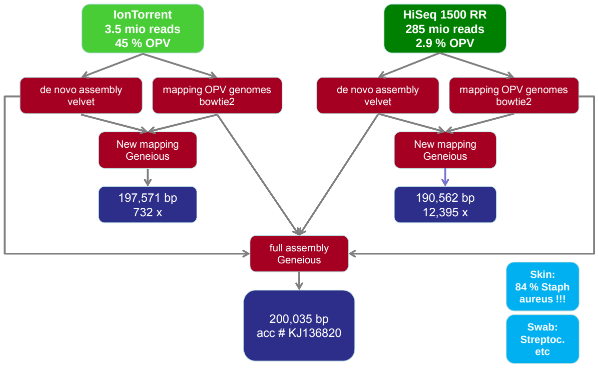

Phylogeny

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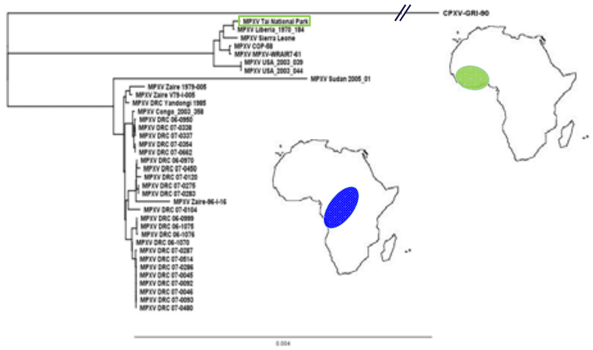

NJ tree

Discussion

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- First report of a wild-living monkey fatally infected with MPXV
  - Infant animal! Impact of age on fatal outcome?
- MPXV not definitely proven as causative agent
  - Individual constitution?
  - Bacteremia?
    - 84% reads from skin sample Staph aureus
    - Several bacteria in throat swab (Staphylococci, Streptococci)
- MPXV isolate clusters to West African strains of lower virulence
  - Virus strain host species combination !
- July 21, 2014, Yaoundé, Cameroon: "At least six chimpanzees in an enclosed sanctuary have been tested positive for monkeypox, according to (OIE)."

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- One Monkeypox case from Ivory Coast 1971 (proven by epi data)
- No human or animal infections observed in TNP recently
- What is the source of the infection?
  - Mangabeys usually live on fruit
- Non-human primates can serve as indicators for the presence of specific pathogens in certain regions
- Bush meat complex of problems (Ebola outbreak!)