Consortium Application within the Funding Activity „Nationales Forschungsnetz zoonotische Infektionskrankheiten“

SYNOPSIS	
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Title of the Consortium	Risk assessment in pre-pandemic respiratory infectious diseases
Acronym of the Consortium	RAPID
Addressed zoonotic infectious disease and pathogens	Preparedness research against prepandemic respiratory infections, using MERS-coronavirus as the main study subject
Structure of the Consortium	*Projects proposed by academic medicine and public health institutions* D. Muth, C. Drosten, Institut für Virologie, Bonn* A. von Brunn, Max von Pettenkofer Institut München S. Hippenstiel, Charité and T. Wolff, Robert Koch Institut, Berlin* *Projects proposed by academic veterinary and animal health institutions* F. Weber, Institut für Virologie, Gießen (with contribution by J. Ziebuhr) V. Thiel, Institut für Virologie, Universität Bern/CH* A. Volz, G. Sutter, Institut für Virologie, LMU München V. Herder, W. Baumgärtner, A. Osterhaus, Tierärztliche Hochschule Hannover U. Wernery, Central Veterinary Research Laboratory, Dubai* *Projects proposed by fundamental research entities* A. Karlas, Max Planck Institut für Infektionsbiologie Berlin S. Pöhlmann, Deutsches Primatenzentrum Göttingen *Projects proposed by veterinary and food industries* P. Nagy, J. Juhasz, Dubai Camel Industries and Products *Institutions with roles in public health or cross-border / public animal health
Summary of the Proposal	*Zoonotic viruses that undergo onward transmission in humans via the respiratory route bear a high pandemic potential. Based on MERS-coronavirus, a paradigmatic prepandemic virus, we will collaboratively transform the latest methodology from basic respiratory virus research into a public health laboratory toolbox to assess the pandemic potential of variant and novel respiratory viruses. Utilizing the OneHealth principle for an anti-zoonotic intervention in the MERS-CoV reservoir (camels), we will conduct a vaccine trial in a globally unique study setting in Dubai using a human vaccine developed by DZIF (now in human phase I trial). Our program will integrate German public health and translational research structures into a One Health research resource that comprises the whole chain of emergence of MERS, one of the most severe threats to human health security today.*
Requested Funding for the Consortium	*3.6 Million €*
Requested Funding Period	*5 years*

1. Current Situation

1.1. MERS as a prototype for pre-pandemic respiratory infections

Pandemics pose a considerable threat to societies. Respiratory viruses are predisposed to become pandemic because of their favorable transmission pattern. Cross-host infection of immunologically naïve populations with rapid optimization of viral replication level or biophysical transmissibility is at the origin of all pandemics. In livestock, cross-host adaptive infection can give rise to panzootics.

The Middle East Respiratory Syndrome-coronavirus (MERS-CoV) is a prototypic prepandemic agent for which no approved treatment or vaccine is available. Hospital-based mortality can be as high as 30%. MERS-CoV is acquired as a zoonotic infection upon contact with infected dromedary camels [1]. Limited human-to-human transmission is possible, causing devastating nosocomial outbreaks [2, 3]. In the best-studied outbreak so far, importation of a single case to South Korea caused 5 generations of onward transmission, involving 186 patients (36 fatalities) in several hospitals across the country [4]. The damage to the economy almost led the country into a recession.

Among the major viral epidemics in the past years (MERS, Ebola, Zika), MERS-CoV poses the most continuous threat as it has an active source in a livestock species, and is transmitted via the respiratory route. The establishment of sustained human-to-human transmission would inevitably lead to a pandemic. As we have no data on the variability of transmissibility among different MERS-CoV strains including those in animals [5], the virus is one of the most threatening issues in global health security today.

Several human respiratory viruses other than influenza can be traced to zoonotic origins. Public health was challenged by the severe acute respiratory syndrome (SARS) outbreak that started upon zoonotic acquisition and spread internationally within months in 2002/03. This first pandemic of the new century was stopped only upon concerted, international public health intervention. Among the four endemic human coronaviruses (HCoV-229E, -NL63, -HKU1, -OC43), for at least 2 viruses there is evidence for zoonotic acquisition in historical times, followed by pandemic expansion and establishment in humans worldwide. HCoV-OC43 stems from cattle, one of the main livestock species globally [6]. HCoV-229E seems to stem from dromedary camels just like the MERS-CoV [7]. Several other endemic human respiratory agents can be traced to livestock, including respiratory syncytial-, parainfluenza-, and human metapneumovirus, some adeno- and enterovirus types, and even the measles virus.

These examples, with MERS-CoV on first priority, suggest that it may be shortsighted to focus pandemic preparedness research on influenza only. While drugs and prepandemic vaccines already exist for influenza, we would be entirely unprepared against other viruses widely existing in livestock reservoirs. In addition, the risk of imported, nosocomial outbreaks of MERS poses a novel, actual, and practical challenge to public health.

1.2. Relevance of the topic for public health

Pandemic preparedness planning is among the chief issues in public health nationally and globally. In Europe, preparedness planning is based on Article 4, Decision 1082/2013/EU. There is a clear mandate for ECDC to conduct science-based risk assessment. In Germany, the updated scientific part of the national pandemic preparedness plan ("*Nationaler Pandemieplan*"), published April 4th, 2016, emphasizes the variable spectrum of risks associated with pandemics ("*Preparing a flexible response to different pandemic scenarios").* In the chapter "*Dealing with unpredictable parameters of a pandemic*" the text mentions exactly those tasks that are central to the research agenda of this consortium, including the determination of phenotypic variability of unknown and novel viruses. The *Pandemieplan* also emphasizes the necessity to conduct **science-based risk assessment on national level in Germany**, in addition to international assessments ("*National risk assessments as a basis for measures to be taken*"). MERS is explicitly mentioned.

MERS poses a challenge to national public health due to the continued risk of importation by a large number of healthcare seekers, pilgrims and migrants from the Middle East. Germany has experienced at least three cases of imported MERS-CoV infection since 2012. Each of these cases could have caused an outbreak as dramatic as that in Korea. In all three cases, public health entities were brought to the limits of their capacity with contact tracing, infection control, and risk communication. MERS belongs to those diseases that are most difficult to deal with in the German notification system as the non-anonymized notification implicates a risk assessment by public health entities (criterion “severe threat to the public” according to German infection protection act (IfSG) § 6 Abs. 1 Nr. 5a). Except some methods that are only applicable to influenza, public health laboratories in Germany have no tools to study risks posed by outbreak strains.

Beyond MERS, the assessment and control of prepandemic risks is becoming a growing demand by citizens in western societies. Health security and the protection against epidemic diseases is now being perceived as a societal achievement on a rank similar to consumer protection, food safety, and protection against environmental hazards. However, pandemic preparedness research has almost exclusively focused on influenza, and has suffered from misleading debates about gain-of-function experimental approaches and the idea of “dual use research of concern”. The public health community needs positive, generalizable methodology adapted from basic research that could **advance the capabilities and legacy of both human and veterinary health authorities**. Public health needs fresh approaches to:

-Inform the public regarding risks associated with imported diseases
-Act on an evidence-based foundation in the field of international trade and travel regulations
-Implement and justify control measures against nosocomial outbreaks
-Guide decision making in healthcare policy and economy
-Guide the prioritization and funding of basic and translational research

1.3. The need for interdisciplinary research

1.3.1. The One Health-approach in MERS research

MERS is primarily a human disease causing only mild symptoms in animals. As a paradigm for the OneHealth concept, this human disease would best be controlled by veterinary intervention at the zoonotic source of infection. Conversely, the severe consequences for human health make MERS a significant topic for livestock industries and veterinary medicine. Our project is designed to meet the dual demands in human and veterinary medicine regarding MERS. It is integrated with other BMBF infectious diseases research in Germany to avoid duplications and provide a highly synergistic structure (**Figure 1**).

Veterinarians are integral to the success of this consortium and their contribution is not limited to the provision of samples - this is covered by preliminary work of the coordinator, funded by DZIF. Veterinary expertise will enable a unique OneHealth intervention, using a human vaccine developed in DZIF to study the potential of eradicating the virus from the zoonotic reservoir. Interests in human and veterinary medicine also overlap in infection modeling, systems biology, and virology, but approaches and study subjects are often discipline-specific. Our consortium therefore incorporates groups from veterinary medicine with long-standing experience working on agents relevant for humans (Thiel, Weber, Sutter, Baumgärtner/Osterhaus), as well as human medicine groups working on viruses whose source is in animals (Drosten, Pöhlmann, Karlas, von Brunn, Hippenstiel).

2. Own Previous Work and Infrastructures

Five project partners are former members of the BMBF SARS research consortium that had considerable scientific output and has yielded large parts of the resources we can now rely on. Three partners are members of the DZIF, which provides an infrastructural foundation in human medicine that is integrated into a OneHealth research framework by the present project.

2.1 Viruses, clinical samples, diagnostic tools, reverse genetics

A unique resource enabling the work of the whole consortium has been assembled during several years of BMBF-, DZIF-, DFG-, and European Union-funded work on MERS at the institution of the coordinator. Through successful research collaborations with the Saudi Ministry of Health, the International Livestock Research Institute (ILRI) in Kenya, the Central Veterinary Research Institute (CVRL) in Dubai, the Ministry of Health of Pakistan, as well as the National University in South Korea, the University of Bonn has isolated multiple strains of MERS-CoV from humans and animals, and has collected samples from MERS-infected patients and animals, along with other unique reference reagents (e.g., [1-5, 8, 9]). Based on the literature, these materials may form one of the largest MERS-CoV research collections globally. All relevant diagnostic tools for MERS-CoV infection have been defined by the coordinator, and proven for application in humans and dromedary camels in multiple studies (e.g., [2, 3, 10]. The coordinator´s institution has established one of the few reverse genetics systems for MERS-CoV, enabling the generation of defined virus mutants, reporter viruses, as well as the rescue of live viruses from available sequence information for strains that have not been isolated in cell culture so far.

2.2. Infection systems representing the human host

For the study of virus-host interaction we have the proven ability to generate unique transgenic cell lines covering large groups of interferon effectors (F. Weber, [11, 12]) and transmembrane proteases (S. Pöhlmann, [13-15]). We have hypotheses-free approaches for the discovery of novel virus-host interactions on protein- (A. v. Brunn, [16]) and transcriptional level (A. Karlas, [17, 18]). Both approaches have already been proven to enable the discovery of commonalities in host interaction across diverse viral taxa. Two groups have infection models which, render surrogates of *in-vivo* infection with increasing complexity (**Figure 1**). Lung cell air-liquid interface cultures provide a differentiated respiratory mucosa that can be genetically modified by siRNA and, soon, by CRISPR/Cas9 mutagenesis (V. Thiel, [19, 20]). Lung explant models preserve the real tissue architecture of the lung and provide the typical repertoire of local immune cells of the non-infected lung, which is the ideal starting scenario to model initial infection capability as of interest in prepandemic pathogen scenarios (S. Hippenstiel, [21, 22]). For the ultimate (but limited) validation of findings, we have a marmoset infection model at German Primate Center.

2.3. Prerequisites for OneHealth vaccine studies

Within DZIF, one of the participating groups has produced and pre-clinically validated a recombinant Modified Vacciniavirus Ankara (MVA)-derived live vaccine for MERS-CoV which will enter into human phase I trials starting in 2016 (A. Volz, G. Sutter [23]). We have access to GMP-produced vaccine stocks, and we have already provided proof of concept for efficacy in camels [24]. **A globally unique study setting** is provided by a collaboration between the Dubai Central Veterinary Research Laboratory (CVRL) and the Dubai Camel Industries and Products Ltd. (DCIP), keeping ca. 5000 adult camels and ca. 300 calves per year. Together, we have found these animals to be “reliably” infected with MERS-CoV each year within months after parturition [9, 10]. We will be able to conduct a vaccination and natural challenge trial in this unmatched setting. Drs. Peter Nagy and Judith Juhasz, who have established the unique husbandry at DCIP over many years, are active members of the consortium, overlooking the trial. The pathological work up will be performed in collaboration between CVRL, the top reference institution for camel diseases in the Middle East, and Tierärztliche Hochschule Hannover. Immuno-virological workup will be done in Munich, along with the DZIF human trial.

3. Aims and Structure of the Consortium

3.1 Aims of the consortium

Here we want to study MERS as a prototypic example of a prepandemic respiratory infection and thereby create a new methodological framework for applied experimental risk assessment of outbreak- and reservoir strains by public health laboratories. Extending beyond MERS, we want to create predictive tools to assess pandemic risks posed by prepandemic respiratory viruses in general. As a task addressing intervention, we want to complement the planned DZIF

phase I trials on MERS-CoV vaccination in humans by a relevant veterinary vaccination trial, based on the human vaccine. This work will pilot the OneHealth approach in vaccinology.

3.2 Structure of the consortium

3.2.1. Complementarity with existing infection research structures

Existing structures for pandemic preparedness include health authorities performing disease surveillance in humans and animals (e.g., RKI, FLI), as well as research entities such as DZIF working on novel therapeutic targets and diagnostic tools to fight emerging diseases. **Our consortium will not duplicate the work of RKI, FLI and DZIF**, but develop a research network that will be highly complementary to these structures. For example, while DZIF is strongly focused on anti-infective drugs, the here-proposed program avoids any drug discovery or drug development activities. Moreover, DZIF does not focus on the veterinary field but conducts medical research that complements the here-proposed work into a visible and credible OneHealth environment with unity of purpose.

Figure 1 provides an overview of projects and their fit within a larger context of research structures. Project numbers in the following text are defined in the figure as well as in **Table 1**.

Table 1. Investigators and institutions involved in the consortium

Name (Mentor†)	Affiliation	Responsibility/ Role/ Contribution	Project no.
D. Muth (C. Drosten)	*Humanmedizin Universität Bonn*	Coordination and provision of biological resources MERS-CoV human adaptive capability, linking with public health (Consultation lab for Coronaviruses).	*1**
A. Karlas (T. Meyer)	*MPI für Infektions-biologie Berlin*	*Identification of virus-host interactions on transcriptional level, construction of assay systems*	*2*
A. von Brunn	*LMU Humanmedizin München*	*Identification of virus-host interactions on protein level, construction of assay systems*	*3**
S. Pöhlmann	*Dt. Primatenzentrum Göttingen*	*Cross-host requirements in entry and egress*	*4**
F. Weber, J Ziebuhr	*Veterinärmedizin Univ Gießen*	*Innate immunity phenotype*	*5**
V. Thiel, R. Dijkmann	*Veterinärmedizin Bern*	*Modeling of host transition in the respiratory epithelium*	*6**
S. Hippenstiel, T. Wolff	*Charité and Robert Koch Institute, Berlin*	*Modeling of human lung infection, linking with public health (National Reference Centre for Influenza)*	*7*
A.Volz (G. Sutter)	*LMU Veterinärmedizin München*	*Vaccine-related immune response in camels*	*8*
V. Herder (W. Baumgärtner)	*Tierärztliche Hochschule Hannover*	*Vaccine-related pathology in camels*	*9*
P. Nagy, U. Wernery	*DCIP, Dubai, UAE CVRL, Dubai, UAE*	*Camel vaccination study*	*10 (own funding)*

*Previous members of the BMBF SARS research consortium (2007-2013).
†Mentors mentioned in parenthesis in case of (co-)applications by junior scientists

3.2.2. Evolution of the consortium structure since conclusion of the BMBF SARS project

The groups Drosten (**P1**), von Brunn (**P2**), Pöhlmann (**P4**), Weber (**P5**), and Thiel (**P6**) have successfully collaborated during the previous BMBF SARS consortium. However, scientific progress and changes in epidemiology as well as research structures have triggered considerable reorganizations of the prior consortium, along with a re-targeting and evolution of the former work program.

Obviously, the **emergence of MERS-CoV** has triggered major re-orientation towards a novel virus system for all groups. The focus on bats has been discontinued in the present work program because our own work, mainly funded through the BMBF SARS project, has shown that MERS-CoV has no direct source in bats [8]. Moreover, while the prior project involved a major focus on the detection and sequencing of novel viruses in the animal reservoir, we have now secured an infrastructural funding for this type of work through DZIF at the coordinator´s lab (**P1**). During 2012-2015 we have sampled MERS-CoV variants from humans and camels,

which are now available for study in the present project. One of the most important outcomes of our preliminary work on MERS has been the insight that knowing the genome sequence of outbreak-associated MERS-CoV contributes little to risk assessment. Instead of further screening of genetic diversity, **P1** will therefore focus on approaches to take **functional viral diversity** into infection models throughout the consortium, mainly based on reverse genetics.

Conceptual features that have been maintained include our reliance on hypotheses-generating studies of virus host interaction, which has proven highly successful during the SARS project [16]. The yeast2hybrid group (**P3**) has therefore been complemented by the inclusion of an additional systems biology group [17, 18], employing most recent technologies in transcriptional knockdown screening via CRISPR/Cas9 approaches (**P2**). The finding of transmembrane proteases as important mediators of coronavirus (and other enveloped viruses´) entry and egress was a major achievement in the SARS consortium that will be intensified as a central field of interest here [13-15] (**P4**). While a multitude of novel insights has been gained on innate immunity, the anti-Coronavirus effectors are still largely unknown [11, 25-28]. Because of the cross-system relevance and commonalities of IFN-induced antiviral effectors [29], we will intensify our engagement in this area (**P5, P6**). The recent acceptance of chairs of virology by both project leaders (F. Weber, Gießen and V. Thiel, Bern) exemplifies the importance of innate immunity research in veterinary medicine.

Due to limitations in **MERS-CoV disease modeling**, infection and pathogenesis studies in mouse models have been discontinued in the present project. Instead, one of our partners will provide novel technology to model epithelial infection (**P6**) [19, 20], and a new group with a human pathogenesis research and **clinical** background was included to provide a unique human lung infection model [21, 22], which we hope will more appropriately represent MERS infection in humans (**P7**). Criteria for **virulence scoring** will be an essential outcome from this model. Moreover, the recent inauguration of the **P4** project leader as the head of the Infection Biology Unit at the German Primate Centre provides the unique opportunity to take selected research outcomes into a primate model proven for MERS-CoV infection (*C. jacchus*) [30].

While the previous project was intensely focused on antiviral target identification, we are entirely stopping our engagement in this field because of the eminent role of **DZIF** in the area. On the other hand, DZIF has yielded a convincing vaccination approach for humans that we will use for veterinary application in this project, aiming at a **OneHealth vaccination** concept. Two novel groups (**P8, P9**) [23, 24], as well as an important overseas partnership with two groups in **Dubai** were added to the consortium for this purpose [9, 10].

3.2.3. The collaborative structure within the consortium

P1 will provide viruses, clinical samples and diagnostic tools to all groups. Both systems biology groups (**P2, P3**) can mutually confirm each other´s outcomes. **P2** is highly interlinked with data from earlier virus-host interaction screening projects, enabling a cross-identification of broad motifs in virus-host interaction.

P4 and **P5** define our major preliminary hypotheses on virus-host interaction. Because many host restriction factors in entry and egress are interferon-sensitive genes, there is a natural and documented collaboration between these two projects. **P4** and **P5** will take novel findings from **P2** and **P3** into their cellular assay systems and contribute them the the risk assessment platform in the second stage of the project (**Figure 1**).

Infection models in projects **P4** to **P7** form a **system of host surrogates of escalating complexity (Figure 1)** - another natural field of collaboration. Results from projects **P2-P5** will be taken into artificial mucosal models by project **P6**. These models are amenable to genetic manipulation like cell-based assays, and provide for the first time a methodological basis for the confirmation of cell culture-based findings in more realistic models of epithelial infection. Results from the epithelial model are challenged by full lung tissue infection in **P7**. Very selected scenarios can be taken into a primate model in **P4**.

Also the vaccine approach is designed to involve natural fields of collaboration while being structurally complementary to existing research. The MVA-based MERS-CoV vaccine was developed by **P8** in DZIF, and will be studied under the future DZIF work plan exclusively for human application. For our OneHealth vaccination approach we will link some of the best camel disease experts globally (**CVRL, DCIP**) with those two German groups that have jointly provided a first proof of concept for the application of the MVA vaccine in camels (Hannover and Munich, both in **P8**).

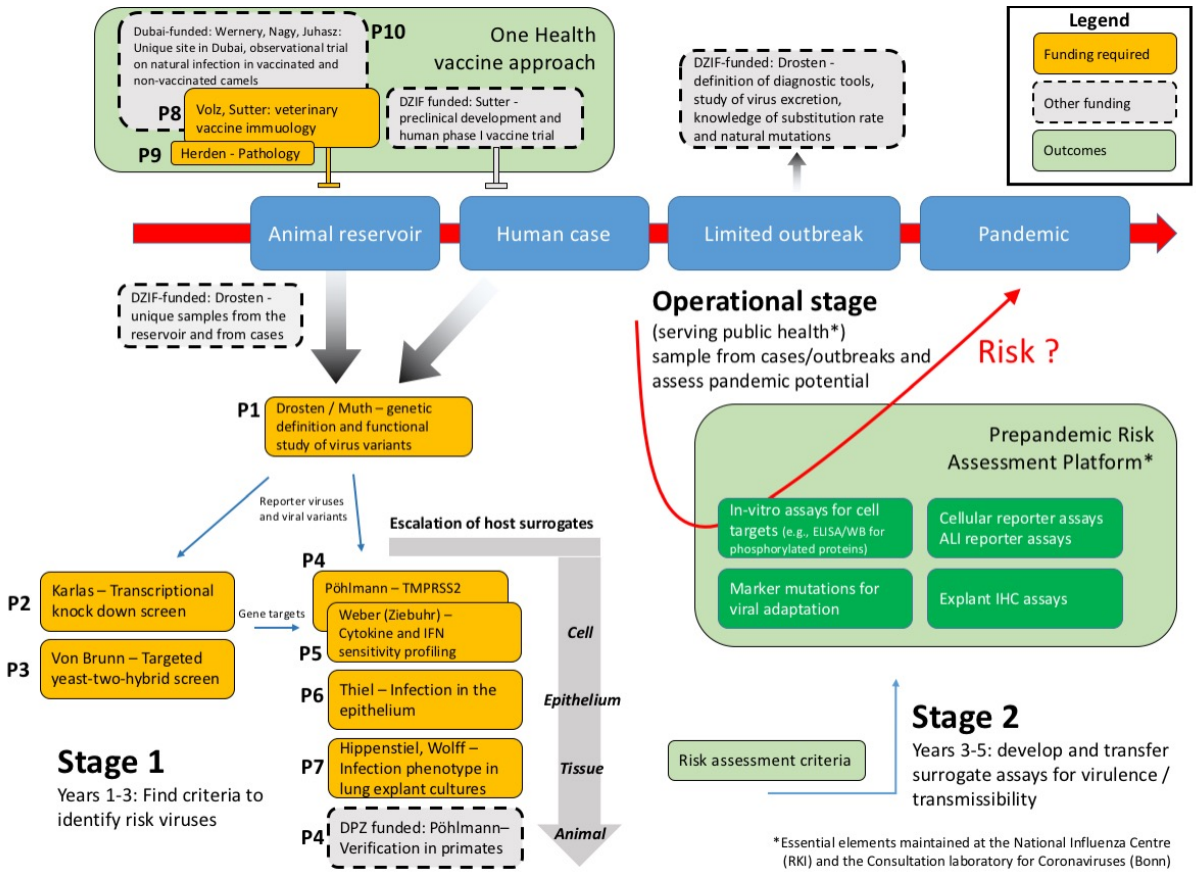


Figure 1. Project partners, organization, and complementarity of the proposed work. *Green fields symbolize the 2 major project outcomes (Prepandemic risk assessment platform and OneHealth vaccination approach). Orange fields are projects to be funded under this proposal.Grey fields are contributions funded by external sources (DZIF, German Primate Center, Dubai Ministry of Climate Change and Environment).*

3.2.4. Plans for continuation and interlinking with public health

The official platform linking the academic research community to the national public health structure in Germany is the **Network of Consultation Laboratories and Reference Centers**. The network is organized by a dedicated commission located at the Robert Koch Institute and reporting directly to the Ministry of Health. Our consortium is represented by **two independent links** in the Network. First, the most important endpoint in the experimental risk assessment platform (lung model, **P7**) is based on a long-standing collaboration between Charité and RKI. The head of the influenza /respiratory virus unit of RKI that houses the National Influenza Centre (T. Wolff) is co-project leader in **P7**. Second, the coordinator of the consortium (project **P1**) heads the Consultation Laboratory for Coronaviruses in Bonn, reporting directly to RKI.

The involvement of several of our partners in DZIF will provide additional infrastructural support for continuation of our work after the end of project funding (**Operational stage, Figure 1**). DZIF is particularly interested to strengthen its collaborative relationship with human and veterinary public health entities and has accepted RKI and FLI as partner institutions. The outcome of our project will provide an important bridge between DZIF institutions and RKI in particular.

Of note, we are **not including FLI** as the top veterinary public health institution in this consortium, as there is a **DZIF project on virus discovery in livestock funded at FLI, TiHo Hannover, and the University of Hamburg**. It is highly interlinked with the present project as C. Drosten coordinates the virus diversity research in the DZIF Emerging Infections unit. We do not require additional virus samples from livestock for the present project.

3.3. Work Plan

3.3.1. Work aimed at the establishment of a Prepandemic Risk Assessment Platform

To establish the prepandemic risk assessment platform we will use MERS-CoV as a common study subject. We will create a common line of work by studying a defined set of MERS-CoV variants in a coordinated way, with useful timely overlap between projects. This set of viruses (to be extended in later phases) includes MERS-CoV reference strain EMC, MERS-CoV delta p4 (a variant with a deletion in protein 4 encountered during an outbreak in Jordan [31]), as well as MERS-CoV D510G (a variant encountered in two patients during the Korean outbreak [4]). Based on known protein functions and preliminary experimental data, both viral variants are highly likely to be attenuated (refer to **P1** description).

Viruses will be constructed by **P1** using reverse genetics and distributed to projects for the establishment of cellular and more complex infection models. The chosen viruses are particularly useful to initiate work regarding virus entry (**P4**) and cytokine profiling (**P5**) because the D510G variant is expected to be deficient in receptor binding and the p4 variant is expected to be deficient in the induction of the interfering response. For several projects, **P1** will generate reporter viruses based on reference strain EMC/2012 expressing green fluorescent protein (GFP) or luciferase. Project **P2** will use the GFP virus to establish CRISPR/Cas9 screening for MERS-CoV cellular interactions. **P3** will receive an error-corrected MERS-CoV cDNA clone for the establishment of the yeast two hybrid system. GFP-containing viruses will also be provided to **P6** and **P7** in order to determine replication phenotypes in epithelial and lung explant models.

The cell-biological projects **P4** and **P5**, as well as partially the epithelial modeling project **P6** will take over findings from the systems biology projects (**P2** and **P3**) and introduce these findings as additional targets into their cellular assay systems. Based on their experience, we will initiate the construction of risk assessment assays by **P6** and **P7**. This work will typically involve the introduction of knockouts or overexpression in **P6 (**enabled by **P2)**, as well as the establishment of marker-specific immunohistochemistry in (basis for objective virulence scoring) in **P7.**

The viral risk assessment platform has to rely on the availability of live virus isolates. Therefore, as an essential component of the platform, several projects in the consortium will generate tools to enable better success in the isolation of live viruses in case of outbreaks. First, through collaboration between **P1** and **P4** we will generate cell lines that are particularly competent for the isolation of viruses (refer to **P1** description). Second, **P6** will generate interferon-deficient epithelial cultures, including indicator gene-expressing epithelial cultures, which can facilitate the isolation of viruses that are refractory to isolation in normal cell cultures or that do not generate cytopathogenic effects. These methods will be applicable to a large range of pathogens and can thus be provided to a broad community of zoonotic diseases researchers.

During a second working period (**Figure 1),** the application of assays will be validated for further variants of MERS-CoV, and further for other coronaviruses, influenza, paramyxo-, and enteroviruses. For MERS-CoV, **P1** will provide genetically defined variants which will have been rescued by reverse genetics up to this time. In particular, these variants will include phylogenetically outlying viruses which have been detected and sequenced from camels in Africa, but not isolated in cell culture. Testing of these viruses within the whole consortium will provide insight into risks associated with the existing zoonotic reservoir, and will also provide important data regarding the necessity to adapt vaccines in the future. Using the infection models created in **P6** and **P7**, as well as (ultimately) the primate infection model, we will be able to predict whether and to what extent these variants can affect humans, compared to prototype viruses. Going beyond the coronaviruses, we will test a large repertoire of defined respiratory viruses that are being maintained in the laboratory of J. Ziebuhr at the department of Medical

Virology in Gießen (**P5**). **P5** will thus promote another human-/veterinary medicine collaboration locally (Weber/Ziebuhr).

Last, we will implement frontline assays designed in this consortium at RKI to make them available for public health in the case of an outbreak (operational phase, **Figure 1**). Only the two projects establishing and maintaining the Risk Assessment Platform (**P1** and **P7**) will be active in the **last year of funding**, dedicating their work to technology transfer. An additional essential component of technology establishment in **P7** will be to establish freeze-preservation of lung explant sections to enable immediate assessments including **virulence scoring** of pre-pandemic viruses. Also this methodology will then be available at RKI. To facilitate transfer of these tools to other public health entities (*Landeslabore*), we will pilot a series of training and tech transfer workshops that can be continued by RKI within the Network of Consultation and Reference laboratories.

3.3.2. OneHealth vaccine study

We have the opportunity to conduct a camel vaccination study in a unique and large industrial camel husbandry. On site, veterinary supervision will be provided by DCIP (P. Nagy, J. Juhasz). Immediate microbiological follow-up will be provided by CVRL.

In 2014 and 2015, we have seen that camel calves will be infected within 6 months post parturition at 100% rate. To confirm the timing of natural infection in the herd once again, **P1**, **P8, P9,** and **P10** will conduct another observatory trial of natural infection during the first year. In Munich, **P8** will complete the methodological repertoire for the measurement of cellular immunity in camels during year 1. In collaboration between CVRL and Hannover, logistics and personnel exchange will prepare pathological studies along with the vaccination trial. The actual vaccination and challenge study will be conducted during year 2 by vaccination of 3 groups of animals using 3 different regimens, as well as a control group (refer to **P8** description). Microbiological follow-up (RT-PCR, serology – provided by **P1**) will be done in CVRL. Samples will be shipped to Munich for advanced viro-/immunological testing. During the trial, three animals per group will be dissected under study conditions, assisted by a study pathologist from Hannover (**P9**). Data validation will be conducted during year 3 by all groups collaboratively. Depending on the results, vaccination success upon single vaccine dosage will be studied during years 3 and 4.

3.3.3. Self-assessment regarding dual use research of concern (DURC)

The work proposed in this project does generally not meet the criteria for dual use research of concern (**Table 2**). There is one aspect in one project (**P1**) that involves experimental adaptation of a severely attenuated MERS-CoV to replication in human cells. A specific self-assessment for this work provided in **P1** explains why DURC potential should not be applicable.

Table 2. DURC potential

Sub-Project no.	Method	Material	Aim	DURC potential (yes/no)
1-9	Laboratory studies	Cell and tissue cultures	Studies of virus-host interaction and adaptation of an attenuated virus	No
4	Animal study	marmosets	Studies of natural infection	No
10	Animal study	camels	Vaccination with natural exposure	No

3.4 Added value of the joint work

For public health, we will provide a laboratory toolbox for virus risk assessment that will be available through RKI to federal public health laboratories (*Landeslabore*) and other members of the Network of Reference Centres and Consultation Laboratories. This toolbox will be based on a huge variety of methods from various basic research groups. As the effort to transform basic research methodologies into public health applications does not create direct benefits for

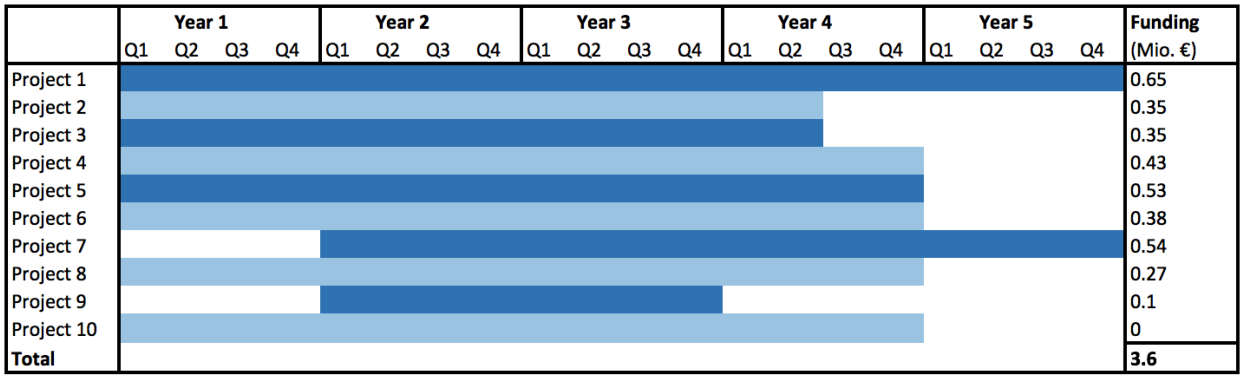
academic research, the work can only be afforded through coordination and appropriate funding. We emphasize that we understand our consortium as a public health research network whose mission should complement but not overlap the sovereign duties (*Amtsaufgaben*) of health authorities of the federal states (*Bundesländer*) and the Robert Koch Institute. The here-proposed work will not duplicate the engagement and funding of federal and national authorities in pathogen surveillance, disease notification, public involvement and risk communication.

For the wider OneHealth research community, the fundamental difficulty to find adequate infections models for respiratory diseases will make our outcomes relevant beyond virology. As we look at hypotheses regarding virus-host interactions in a cross-sectional approach, the possible identification of common cellular targets will be useful for research on pathogens not investigated here. All project partners will be members of the German Research Platform for Zoonoses to ensure outreach to the broad zoonosis research community. Consortium partners will gain access to established networks and comprehensive databases. The consortium will also apply for membership in the TMF e.V. to get access to efficient infrastructures for the promotion and organization of networking activities by participating in the AG Zoonoses and Infection Research.

3.4.1. Promotion of career development of female scientists

We are committed to increasing the representation of women in academic research. Through our program, three female junior scientists will have the opportunity to conduct their first independent research projects necessary to establish their own groups: D. Muth (**P1**), A. Volz (**P8**) and V. Herder (**P9**).

4. Financial summary (in Euros) and Time Schedule



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Project No.	**1**
Title	Functional diversity of circulating MERS-CoV variants
Principal Investigator	*Dr. rer. nat. Doreen Muth (PI for the present project)* *Prof. Dr. med. C. Drosten (Consortium Coordinator)* *Institute of Virology* *University of Bonn Medical Centre*
Contribution to the One Health approach	*Enabling studies of viral diversity existing in the animal reservoir and affecting human health*
Abstract	**Intention and benefit for the consortium and for public health** *This work package steers the collaborative workflow of the consortium. Essential virus isolates will be provided. Mutations of interest will be constructed into a reference MERS-CoV backbone by reverse genetics. Viruses not isolated in cell culture will be rescued de novo. For systems biology projects (**P2, P3**) and projects employing high-throughput assays or complex infection models (**P4, P5, P6, P7**), reporter viruses will be provided. Together with **P4**, a highly susceptible virus isolation cell line will be developed to enhance virus isolation capabilities in the prepandemic risk assessment platform. Diagnostic methods will be provided to **P8**, **P9** and **P10**.* *In an own research area, the project will determine whether MERS-CoV can increase its replication level upon adaptation to human cells. We will create a MERS-CoV that is deficient in its error correction function and thereby shows an increased error frequency that attenuates the virus, but enables it to adapt faster to cells than wild type. We will conduct serial passaging in cell cultures, air liquid interface cultures (**P6**), as well as lung explant cultures (**P7**). If MERS-CoV has adaptive capability to optimize human infection, the attenuated virus will show a limited recovery of replication level after passage. In spite of experimental adaptation, the virus will continue to be severely attenuated due to the lack of essential enzyme function.* **Technical highlights, novelty, uniqueness** *Our virus isolates and clinical samples may form one of the largest MERS-CoV research collections worldwide. We have independently established reverse genetics for MERS-CoV. This combination of clinical and molecular MERS-CoV resources is globally outstanding.* **Expected outcomes and perspectives** *Our project will generate novel tools for virus characterization and address one of the most urgent questions in MERS-CoV applied research (adaptive capability to humans, with identification of human-adaptive mutations for sequencing-based surveillance). Through coordinating the consortium, we will establish an essential set of laboratory-based risk assessment in National Influenza Centre at RKI, as well at the Consultation Laboratory for Coronaviruses.*

1 Working hypothesis and Research question(s)

Surveillance of zoonotic reservoirs and outbreaks by virus detection and sequencing can provide a first assessment of the genetic diversity of viruses. However, the possible spectrum of virulence can only be forecast by characterization of the diversity of pathogen functions. Public health suffers from a gross lack of capability to assess the spectrum of virulence of pre-pandemic viruses in outbreaks and zoonotic reservoirs.

It is the central **hypothesis** of this work package, relevant to the overall project, that not all variants of MERS-CoV are the same. We expect a relevant diversity of virulence in viral variants that have either been encountered in earlier human outbreaks, or that have been detected (but

not functionally characterized) in the camel reservoir of MERS-CoV. Phenotypic diversity will manifest itself in variable replication level, immune interference, and host factor utilization. Moreover, we expect that MERS-CoV is still able to optimize its replication in human cells by adaptive mutations. We want to identify mutations critical and typical for human adaptation that can be screened during sequence-based zoonotic reservoir surveillance.

2 Own previous work and publications

Our group has pioneered public health laboratory research to understand basic features of MERS epidemiology by defining diagnostic tools and using them on defined cohorts both in humans and animals. We have contributed essential parameters to the understanding of the natural history of MERS, such as the transmission in non-hospital settings [1], the rate of infection in the normal population [2], virus shedding in hospitalized patients [3], as well as studies of virus diversity in the reservoir and virus evolution in hospital outbreaks. We have also defined major epidemiological features of MERS infection in camels, including the history and geographic range of seropositive animals, the seasonality of transmission, as well as the age at which animals are typically infected. Based on our clinical and epidemiological studies, we have assembled a unique collection of clinical samples and virus materials from humans and animals (e.g. [3, 4]). Original clinical samples are preserved in a way to make them amenable for further study and sharing within the consortium. Finally, we have provided essential contributions to the understanding of viral functions such as receptor usage and interferon interference. In particular, we have identified protein 4a as a MERS-specific interferon antagonist [5]. In our reverse genetics system for MERS coronavirus the viral genome is cloned in a BAC vector which can be mutated by recombination mutagenesis (**Figure 1**). This system enables the cloning of genetically defined viruses, the targeted introduction of mutations, as well as the rescue of viruses that have not been isolated in cell culture.

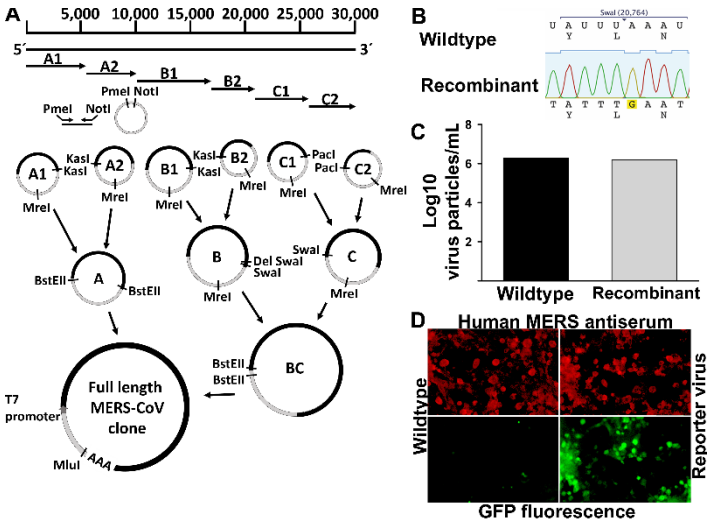

Figure 1: A) Cloning strategy for the MERS-CoV reverse genetics system. B) A point mutation deleting a natural SwaI restiction site serves as distinctive feature between wildtype MERS-CoV and recombinant virus, which both replicate to similar titers (C). D) Only the reporter virus but not the recombinant wildtype virus shows green fluorescence. As a control, infected cells were stained using a human MERS antiserum.

2.1. Five project-relevant references

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3 Work plan including milestones

3.1. Provision of viruses and establishment of Prepandemic Risk Assessment Platform

To enable work in the consortium, we will generate or isolate viruses with defined genetic background, characterize them in cell culture, and provide them to partners for in-depth

characterization using their respective infection models and assay systems. The following viruses are foreseen to be provided, but can be replaced if other MERS-CoV variants emerge during the project.

3.1.1 Recombinant wildtype MERS-CoV and MERS-CoV reporter viruses

The recombinant MERS-CoV reference strain EMC (rEMC) has been rescued by reverse genetics. The genome was corrected to represent the original virus as sequenced from the MERS index patient (the virus isolate available in most laboratories contains cell culture-based mutations). rEMC based reporter viruses expressing green fluorescent protein (GFP), luciferase, or other reporter genes will be created and validated for wildtype efficient replication before they are provided to partners (rEMC with GFP already rescued, **Figure 1D**).

3.1.2. Major outbreak strains: Jeddah 2014 and Korea 2015 (including a Korean variant)

It is among the central aims of the consortium to understand whether or not the severity of major hospital-based outbreaks was determined by virus functions. We will generate plaque-purified and sequenced viruses representing the 2015 Korea outbreak strain [6] and for comparison the 2014 Jeddah outbreak strain [7] (**milestone 1 after 6 months**). Both viruses have been primarily isolated in our laboratory and will be studied using the whole range of infection models provided by the consortium. In collaboration with partners **P6** and **P7**, we will find out whether these outbreak viruses spread more efficiently than other viruses in epithelial cell culture models as well as human lung explants. Depending on initial findings, we will take the viruses into the *Callithrix* model in collaboration with **P4**.

A spike protein variant (D510G) was encountered in two patients during the Korean outbreak. Based on known protein structure [8] and preliminary experimental data, the observed mutation in the receptor-binding domain of the spike protein should destabilize receptor binding and therefore attenuate replication. Recombinant MERS-CoV carrying this spike mutation will be generated and forwarded to partners **P4** and **P6** (**milestone 1 after 6 months**).

The Korea 2015 virus is derived from a natural recombination event [9]. To compare the Korea virus with its precursors, we will generate plaque-purified and quantified viruses representing the donors for the recombinant virus lineage. In case we find that the 2015 Korean virus is more replicative or more virulent than its lineage precursors, we will re-create the recombination event that determined the formation of the Korean 2015 lineage (**milestone 2 after 2.5 years**). Testing will be in collaboration with **P6**, **P7**, and optionally, **P4**.

3.1.3 Potential IFN-based attenuation in the Jordan outbreak, 2015

This virus represents a variant with a 16 amino acid deletion in protein 4a that was encountered during an outbreak in Jordan in 2015 [10]. It spread to an unknown number of patients, but seems to be extinct. Protein 4a (p4a) is an interferon induction antagonist [5]. The deletion likely renders p4a nonfunctional and should thus attenuate the virus.

The naturally observed mutation will be engineered into the backbone of rEMC. In case initial replication experiments will confirm attenuation, we will determine whether, according to our hypothesis, the attenuation is due to increased interferon induction. The virus will be replicated in A549 cells competent for IFN induction, and IFN beta gene expression will be measured by RT-PCR. Replication will be compared in Vero delta lambda cells, a cell line completely devoid of any antiviral interferon induction (see below). The virus will be forwarded to partners **P5** and **P6** for further functional study (**milestone 2 after 2.5 years**).

3.1.4. Reservoir-borne viruses (Niger, Nigeria, Burkina Faso)

Several phylogenetic outliers to the known epidemic MERS-CoV lineages have recently been described in African camels [11]. We have agreed to collaborate with the originators of those viruses (group of Professor Malik Peiris, University of Hong Kong) to rescue MERS-CoV with spike protein variants and other major genomic differences observed in these viruses (unpublished/personal communication by M. Peiris). To determine potential serotype variability, we will conduct cross-neutralization tests using available human and camel sera from Saudi Arabia exposed with typical circulating viruses [2], as well as human and camel sera from Africa [12, 13] that have most likely been exposed with outlier viruses (**milestone 3 in year 4**).

3.1.5. Generation of a cell line with increased virus isolation capacity

The risk assessment platform created in this project will depend on the availability of live virus isolates. We have already generated Vero cells in which the natural IFN beta deficiency has been complemented by a knockout of the complete IFN lambda locus via CRISPR/Cas9. These Vero delta Lambda (Vero_dL) cells replicate many viruses including coronaviruses ca. 10-fold better than normal Vero cells (S. Hauka/Drosten group, unpublished data).
Many enveloped viruses use transmembrane proteases for entry and egress *in-vivo*, but have to rely on auxiliary proteases in cell culture (see **P4**). Only few culture cell lines have maintained transmembrane protease expression, e.g., Caco-2, a cell line that is particularly permissive for MERS-CoV isolation even though it is not interferon-deficient [4]. Because Vero cells do not express TMPRSS2, transgenic expression of this protease in Vero_dL should yield an exceptionally virus-permissive cell line by combining pan-IFN deficiency and full viral protein processing capability. Vero_dL cells expressing TMPRSS2 will be generated in collaboration with **P4** to add a unique virus isolation tool to the Prepandemic Risk Assessment Platform (**milestone 4, end of year 3**).

3.2. Determination of adaptive capability of MERS-CoV to humans

Whereas MERS-CoV is able to replicate in humans, it is not efficiently transmitted. One of the most important questions in pandemic preparedness research regarding MERS is the potential of the virus to further adapt to humans. Whereas our own studies have not shown signs of viral adaptation during the Korea outbreak, adaptation is a stochastic process that may or may not happen during limited outbreaks. Only systematic experimental adaptation in the laboratory can predict adaptive capability. However, as coronaviruses carry error correction enzymes including an endonuclease and a 3´-exonuclease (ExoN), experimental adaptation to cell culture via drift mutations is difficult to achieve for coronaviruses, compared to other RNA viruses. Serial passage will often lead to mutations that are not controlled by error correction enzymes, in particular deletions of non-essential accessory genes which may lead to a loss of function over passages, instead of an adaptive gain of function.

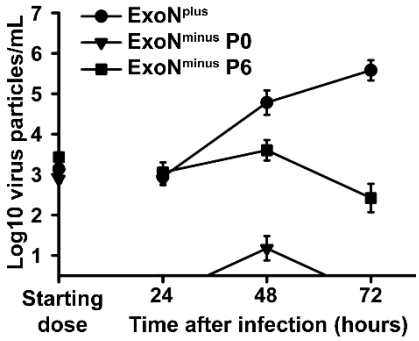


Figure 2: Replication of wildtype (ExoN^{plus}) and exonuclease-deficient (ExoN^{minus}) virus in an inappropriate host cell line. Inactivation of the 3'-exonuclease error correction enzyme (ExoN) leads to impaired virus replication (P0) that covers upon 6 adaptive passages (P6). Even after adaptation, the ExoNminus virus remains attenuated as compared to the non-adapted wildtype virus.

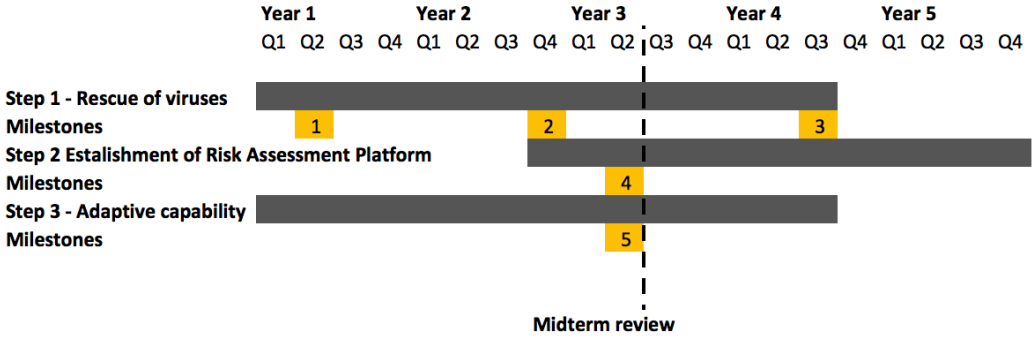
To enable systematic *in-vitro* adaptation experiments, the main error correction enzyme ExoN can be inactivated by a combination of point mutations (D90A/E92A) [14]. For SARS-CoV, we have already generated an ExoN-deficient virus (ExoN^{minus}) which can indeed be adapted to cell cultures in a host transition scenario (**Figure 2**). Without adaptation, ExoN^{minus} replication is considerably impaired as compared to wildtype SARS-CoV because of the essential function of ExoN for maintaining genome stability during replication. Adaptive passaging leads to adaptive mutations and a recovery of replication level, even though wildtype replication cannot be reached because of the ExoN deficiency (**Figure 2**).

Here, we will construct a MERS-CoV ExoN^{minus} variant (**milestone 5, year 2.5**) and conduct serial passage experiments in human lung cell lines (A549, CaLu3). We aim for six to ten passages. The last passage will be quantified and used for comparison to the original virus regarding replication efficiency. If the passaged virus population exhibits improved replication kinetics, we will determine the responsible mutation/s by sequencing. We expect that over 6-10 passages an adapted and improved genome will be enriched to saturation in the population. In collaboration with project **P6**, we will test whether MERS-CoV ExoN^{minus} can replicate in and possibly adapt to ALI cultures. The original MERS-CoV ExoN^{minus} virus as well as the passage-adapted variants will be infected in lung explant cultures in collaboration with project **P7**. We hypothesize that the effect of adaptation will become visible in lung explants by an increase of the number and/or types of cells infected, as well as in replication level. During later stages of the work, we will reconstruct individual mutations into an rEMC- ExoN^{minus} backbone to map phenotypically relevant mutations and thus identify risk markers for virus surveillance.

DURC self-assessment

In spite of the gain-of-function approach in these experiments, we believe that we will not create viruses with increased virulence and DURC potential. Inactivation of the exonuclease renders the virus severely attenuated as compared to wild type virus even after adaptation (**Figure 2**). We do not plan to re-engage the ExoN function in adapted viruses. We expect a natural reversion to be very highly unlikely because of the necessity to revert two amino acids.

3.3. Gantt chart



4 Contribution to the consortium

This project is the coordinator´s work package that collaborates with all other projects. The most important contribution to all projects is the provision of defined virus variants and clinical samples. Clinical samples comprise original respiratory specimens from ca. 40 MERS-infected patients (fatal to mild courses), sera with defined antibody levels (IgG and IgM, known neutralizing titers), respiratory samples with known IgA concentrations, as well as respiratory and fecal samples from camels. 30 virus isolates (28 from humans, 2 from camels) are available. Recombinant viruses with defined mutations are available or will be constructed during the project. Partners **P4**, **P6** and **P7** provide infection models for the evaluation of initial findings of replication phenotypes made here.

5 Quality assurance, standardization, data sharing

Consultation Laboratory for Coronaviruses run by our group is part of our institute´s quality management system (whole process certification of Universitätsklinikum Bonn). All methods belonging to the Prepandemic Risk Assessment Platform will be included.

6 Ethical and legal and considerations

There are no animal experiments involved in this work package. Ethical consent and clearance for scientific study of samples and isolates has been granted by the KSA Ministry of Health, and the Dubai Ministry of Agriculture via CVRL.

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8 Financial summary (in Euros)

Personnel for 5 years			
Position / Salary Group	**Total Budget**	**Duration (months)**	**Tasks / Justification**
Doreen Muth: Technician, full position, TV-L E8	*227,917*	*60*	*Doreen Muth (PhD on CoV reverse genetics 2014) will run this project to start her first group after returning from maternity leave in January 2017. She will be on a staff contract (75%, reduced work time) and will require a full technician´s position to complete the laboratory work. She will not supervise a PhD student because of her reduced work time, but will plan the work for the technician and work in the lab herself.*
Christian Drosten: 60% salary for a an academically trained project administrator	*190,368*	*60*	*The person will organize the many tasks associated with the provision of research materials to and from consortium members (logistics). All communication issues (network of consultation and reference laboratories, media, other BMBF networks, Zoonoseplattform, DZIF, presentation of the consortium at conferences, including public health meetings) will be coordinated by this person. Experience with research coordination as well as public relations will be required (not a secretary´s job).*
Other resources for 5 years			
Type	**Total Budget**	**Specification / Justification**	
Consumables	*75,000*	*15,000 per year mainly for molecular biology work*	
Animal costs	-		
Equipment	-		
Travel	*10,000*	*Conferences, coordination*	
Other	*40,000*	*30.000 for 5 project meetings, 10.000 shipments*	
Sum: Total Budget:	543,285 (ca. 300,000 for own work, 240,000 for coordination)		
Institutional Overhead:	20%		
Sum: Requested Budget	651,942		

8.1. Note regarding overlap with DZIF

Doreen Muth has received a maternity leave stipend from DZIF for maintaining her work from February to December, 2016 (DZIF funded a technician). The work conducted under the stipend is preparatory for the work proposed here, but will end before the here-proposed project can start. The remaining part of the DZIF work program for Bonn has no overlap with the here-proposed work. This project will help Doreen to further establish her group (see above).

Project No.	2
Title	Identification of host factors by loss-of-function and gain-of-function screens
Principal Investigator	Dr. rer. nat. Alexander Karlas, Max Planck Institute for Infection Biology
Contribution to the One Health approach	The identification of host cell factor requirements is of high importance for the understanding of virus tropism and of the transmissibility of zoonotic viruses to the human host.
Abstract	**Intention and benefit for the consortium** Pandemic preparedness research must involve approaches to predict the compatibility of animal-associated viruses with the human host. Obtaining a complete picture of essential host factors would provide a solid basis for the understanding of viral pathogenesis and transmissibility between host species. **Technical highlights, novelty, uniqueness** Recent RNAi loss-of-function screens carried out at MPIIB and elsewhere have revealed initial insight into the role of **cellular nodes** during intracellular pathogen replication and constitute a starting point for this project. Growing evidence points to **common infection strategies** employed by different viruses. Meanwhile, additional powerful methods for target identification are available, such as the CRISPR/Cas systems that allow sequence-specific shutdown of individual genes, or the corresponding gain-of-function system CRISPRa. These technologies are fully established at MPIIB and will be utilized to identify cellular factors involved in MERS-CoV and other relevant emerging viruses´ replication. **Expected outcomes and perspectives** We aim to (i) deepen our knowledge of host cell factor engagement of medically important zoonotic viruses, such as MERS-CoV,using RNAi and CRISPR/Cas-based screens, (ii) elucidate mechanistic differences and similarities to other viruses/pathogens and obtain a global view of cellular networks and nodes involved, (iii) investigate the transmissibility of model pathogens based on such identified factors and their similarity in different host species.

1 Working hypothesis and Research question(s)

One of the most relevant questions in research on zoonotic viruses is why some hosts are highly susceptible to a particular virus while others are well protected. This consortium investigates the ability of zoonotic, prepandemic viruses to infect humans. For a fundamental understanding of the mechanisms determining cross-host transmissibility it is important to realize that the composition of cellular factors within a given host cell determines the ability of a pathogen to infect it. Based on genome-wide loss-of-function screens for influenza and chikungunya virus infection, our previous findings clearly suggest that host susceptibility does not depend only on surface receptors. We found that even unrelated viruses use similar strategies to exploit their hosts, while other host cell determinants can be highly virus-specific, e.g. those required by avian H5N1 but not by seasonal human pathogenic influenza A viruses (IAV). While former screens at MPI were focused on various IAVs and CHIKV, there is a strong necessity to expand these studies to other relevant zoonotic viruses such as MERS to improve our understanding of the emergence of zoonotic viruses.

Our approach is based on the identification of host cell determinants of infection via the knock-down or knock-in of host cell genes during infection. Recent data from MPIIB using RNA inference (RNAi) suggest that even unrelated viruses use similar strategies to exploit their hosts (Karlas et al, 2016). Meanwhile, new technologies have recently become available, such as the CRISPR/Cas systems, which allows sequence-specific shutdown of individual host cell genes, or the corresponding gain-of-function system CRISPRa (reviewed in *1*) - both of which will be employed in this project. Since CRISPR-based screens can be performed in a pooled fashion,

the hands-on time is greatly reduced compared to RNAi screening, supporting multiple screening campaigns in parallel. It will also enable the use of physiologically more relevant primary cells, and reduce off-target effects. Nevertheless, RNAi screens still have their place, since infection assays in an arrayed format allow the identification of host cell factors that are relevant for the whole replication cycle.

2 Own previous work and publications

We now know that host factors are of crucial importance for an infection to occur – thus host factors also constitute potential therapeutic targets. Using its high-throughput screening facility for a genome-wide screen MPIIB identified many host cell factors required for efficient replication of influenza virus (*2*). Based on these hits, detailed follow-up studies have revealed the involvement of a non-coding host RNA in influenza A virus replication. In addition, MPIIB successfully combined the results of related influenza RNAi screens of other groups in a meta-analysis approach to identify highly robust gene candidates that were subsequently verified experimentally (*3*). However, efficient virus replication is not solely restricted by cellular determinants. Thus, MPIIB investigated the potential risk posed by the 2009 pandemic H1N1 influenza virus by serial passaging in human cells. Strikingly, after several passages a 100-fold increase in replication rate compared to wild type was observed, which was pinpointed to two mutations within the hemagglutinin gene segment (*4*). This example illustrates the impact of single mutations within the virus genome and their importance for transmissibility to new hosts. Within the present project we will therefore utilize different MERS-CoVs to elucidate differences and similarities.in host factor requirements. Recently, MPIIB has performed a global siRNA screen to unravel host cell factors for chikungunya virus, an emerging arbovirus formerly restricted to Africa and Asia, which has rapidly spread to Latin America since 2013. Whereas the initial screen was performed using an siRNA library of approx. 60,000 siRNAs, CRISPR/Cas9 gene knockout was used for the validation (*5*). Based on the hits, appropriate partly FDA-approved 'second-use' compounds were identified that efficiently impaired CHIKV replication *in vitro* and *in vivo*. This approach can also accelerate the discovery of novel host-directed antivirals. Convinced by its potential MPIIB has recently used a CRISPR/Cas9 approach for two pooled screens, and identified factors engaged in the replication of chikungunya and hantaviruses.

Five project-related publications

- Karlas, A. et al (2016) A human genome-wide loss-of-function screen identifies effective chikungunya antiviral drugs. Nat Commun, DOI: 10.1038/ncomms11320
- Wörmann, X. et al (2016). Genetic characterization of an adapted pandemic 2009 H1N1 influenza virus that reveals improved replication rates in human lung epithelial cells. Virology *492*, 118-129.
- Tripathi, S. *et al.* (2015). Meta-and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell Host & Microbe *18*, 723-735.
- Winterling, C., Koch, M., Koeppel, M., Garcia-Alcalde, F., Karlas, A., and Meyer, T. F. (2014). Evidence for a crucial role of a host non-coding RNA in influenza A virus replication. RNA Biol *11*, 66-75.
- Karlas, A. et al. (2010). Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature *463*, 818-822.

3 Work plan including milestones

The work package aims to identify host cell factors relevant for MERS-CoV by combining RNAi and CRISPR/Cas9 screens, and set up CRISR/Cas9 pool screening for new emerging viruses with zoonotic potential.

3.1. Genome-wide RNAi screening for MERS-CoV relevant host cell factors

To identify host cell factors affecting the MERS-CoV infectious cycle, MPIIB will run a global RNAi screen followed by detailed hit validation using additional siRNAs and CRISPR/Cas9-based gene knockouts. Developing suitable assays to detect virus replication is a vital necessity for global analyses. Therefore, during the first phase of this project an appropriate cell line will be selected to allow virus propagation and quantification of MERS-CoV replication. Partner 1 (University Bonn) will provide a recombinant virus containing a GFP reporter (rEMC-O4a-GFP or rEMC-O5-GFP) to enable efficient detection of infected cells based on reporter expression. Alternatively, infected cells could be detected by antibody staining, as used by MPIIB for the

global analysis of IAV specific host factors (*2*). Thus, such screens can be performed for any virus as far as specific antibodies are available. Once suitable cell lines have been selected, functional assays will be set up for the arrayed loss-of-function siRNA screen. Initially, MPIIB intends to perform a MERS-CoV siRNA loss-of-function screen in one cell line and develop a fully automated screening procedure as there are ~60,000 siRNAs to screen. To provide reproducible results, pipetting robots located within BSL 2 and BSL 3 safety cabinets will be utilized, which were successfully employed during the genome-wide RNAi IAV and CHIKV screens. Identified targets will be further validated by (i) additional, independent siRNAs and (ii) CRISPR/Cas9 gene knockouts. As the required gRNAs are expressed by lentiviral vectors, primary cells can be used as a more authentic cell model.
Since this screen will be performed in an arrayed format, factors relevant during the whole replication cycle, influencing e.g. virus entry or budding can be identified.

Milestone 1: HTS assay established (month 9)
Establishment of an appropriate screening assay for an arrayed genome-wide siRNA screen
Milestone 2: List of primary hits (month 20)
Completion of the primary screen to provide a list of pro- and antiviral MERS-CoV factors.

3.2. CRISPR/Cas9 loss-of- and gain-of-function screens for MERS-CoV relevant host cell factors

CRISPR/Cas9 pool screening approaches will be used to identify key cellular pathways required by MERS-CoV. This will not only include gRNAs leading to gene knockout, but also gRNAs that bind to promoter regions to activate gene expression (CRISPRa), enabling pair-wise comparison of activated and blocked cellular factors to reveal their effect on replication efficacy. The library available at MPIIB consists of 195,000 gRNAs inhibiting (*6, 7*) and 270,000 gRNAs activating gene expression (*8, 9*). Screens will initially be performed on a common cell line (used in step 1) to identify overlaps between CRISPR/Cas9- and RNAi-based screening results. Additional screens in physiologically more relevant cells will be used to validate the hits for their importance during MERS-CoV infection. Again, recombinant MERS-CoV that lead to GFP expression in infected cells will be used as a read-out system. Alternatively, e.g. if infection rates of a modified pathogen turn out to be insufficient, antibody-mediated detection of positive cells will be used. Based on the fluorescence signal, cells with low infection levels are separated by flow cytometry. Equipment for cell sorting up to biosafety level 3 (BSL 3) is available at MPIIB. gRNAs causing an altered phenotype (low or high infection rates) will be determined by isolating the genomic DNA of distinct sorted cell pools, followed by next-generation-sequencing (e.g. 'Illumina HiSeq 1500', available in-house). For this, gRNAs lentivirally integrated into the chromosomal DNA will be amplified by PCR, using barcoded oligonucleotides to enable multiplexing (i.e. the combined sequencing of different samples). Since a single Illumina sequencing run normally leads to coverage of 150 million reads per lane, several screens can be analyzed in parallel, reducing costs and processing time. As every gene is covered by multiple gRNAs, this provides an additional layer of confirmation, enabling confident identification of the corresponding genes. Screening efficacy will be validated by analyzing the effect of multiple positive and negative controls and reliability determined by repeating the screens at least twice.
These CRISPR/Cas9 pool screens will reveal pro- and antiviral host cell factors in appropriate cell lines and primary cells. An additional level of information will be achieved by specific overexpression of host cell genes based on CRISPRa.

Milestone 3: List of primary hits based on CRISPR/Cas9 pool screening (month 14)
Completion of the initial CRISPR/Cas9 pool screen.

3.3. CRISPR/Cas9 screens with unknown viruses with zoonotic potential

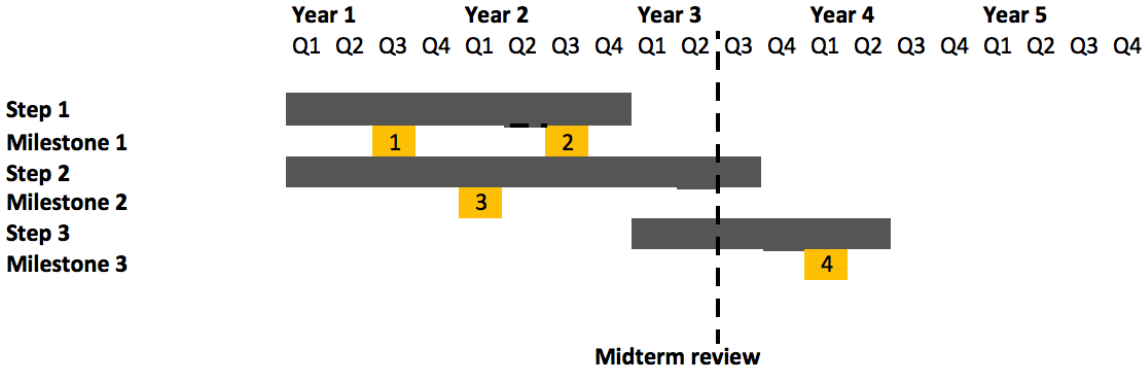
The aim is to establish a CRISPR/Cas9-based pooled screen applicable to newly emerging viruses with zoonotic potential by utilizing the cytopathic effect (CPE) that accompanies many viruses as a read out. This would allow timely screening of newly emerging viruses - without the need for developing recombinant reporter viruses or generating virus protein-specific antibodies. The concept is based on the fact that infected cells will die due to CPE. Thus, cells

expressing specific gRNAs that protect them from infection should be enriched. Next generation sequencing analyses will reveal enriched gRNAs so that underlying genes can be identified. The CPE-based CRISPR/Cas9 screens will initially be validated by performing the first analysis with MERS-CoV. By comparing the resulting hits with cellular target genes identified in step 2, the feasibility of this screening approach will be evaluated.
This approach would enable very fast identification of cellular targets involved in replication of newly emerging viruses, which can be utilized for the establishment of risk assessment assays.

Milestone 4: Establishment of a CPE-based CRISPR/Cas9 screens (month 38)
Proof of principle that demonstrates the usability of CPE-based CRISPR/Cas9 screens

3.3. Overview of milestones and time planning

3.3.1. Gantt chart



3.4. Considerations regarding dual use of research outcomes

This project will generate a list of host cell targets that decrease or increase the replication of MERS after knock-out or knock-in of the corresponding gene. As this approach is based on a modification of the human host cell, it is unlikely that it can be misused for military purposes.

4 Contribution to the consortium

The screening for MERS-CoV essential host factors is strongly facilitated by recombinant GFP reporter and additional mutant viruses provided by **P1**. The identified hits will be provided to all partners to allow (i) detailed characterization of these cellular factors by protein-protein-interaction studies performed by **P3** and (ii) the setup of specific risk assessment assays based on different cellular systems (**P4** and **P5**) as well as on mucosal and human lung infection models (**P6** and **P7**), respectively.

Partner	Project area	Type of collaboration
P1	Infection phenotyping	Receiving of viral strains and mutants
P3	Infection phenotyping	Detailed hit characterization
P4	Risk assessment	Provision of screening results for definition of molecular signatures for pathogenicity
P5	Risk assessment	Provision of screening results for ISG selection
P6	Risk assessment	Provision of screening results for ISG selection
P7	Risk assessment	Provision of screening results for the establishment of risk assessment assays based on a lung tissue model

5 Quality assurance, standardization, data sharing

Screening efficacy will be validated by analyzing the effect of multiple positive and negative controls. Reliability will be determined by repeating the screens at least twice. Hits resulting from the RNAi screen will be validated using additional independent siRNAs, and compared with CRISPR/Cas9 screening results, as well as existing data from related published RNAi screens. This second level of confirmation will lead to highly robust gene candidates. Results will be shared with consortium partners via secured access to the MPI server.

6 Ethical and legal and considerations

Not applicable

7 Key references

1. O. Shalem, N. E. Sanjana, F. Zhang, High-throughput functional genomics using CRISPR-Cas9. Nat Rev Genet 16, 299-311 (2015).
2. A. Karlas et al., Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature 463, 818-822 (2010).
3. S. Tripathi et al., Meta- and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell Host Microbe 18, 723-735 (2015).
4. X. Wormann et al., Genetic characterization of an adapted pandemic 2009 H1N1 influenza virus that reveals improved replication rates in human lung epithelial cells. Virology 492, 118-129 (2016).
5. A. Karlas et al., A human genome-wide loss-of-function screen identifies effective chikungunya antiviral drugs. Nature Communications 7, 11320 (2016).
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8. L. A. Gilbert et al., Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159, 647-661 (2014).
9. S. Konermann et al., Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517, 583-588 (2015).

8 Financial summary (in Euros)

Personnel for 5 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
Postdoc EG14, 60 %	*171,077 €*	*42*	*The Postdoc will design, supervise and analyze the planned screens assisted by a technician. Both will be financed to 60 % by the project and to 40 % by the MPIIB.*
Technician EG 9, 60 %	*64,000 €*	*24*	*Technical assistance for assay development and performance of the screens*
Other resources for 5 years			
Type	Total Budget	Specification / Justification	
Consumables	*108,000 €*	*30,857 € per year in total* Illumina sequencing reagents: 30,000 € Transfection reagents: 30,000 € Cell culture media, plastic ware, antibodies: 28,000 € siRNAs for hit validation: 20,000 €	
Animal costs	-		
Equipment	-		
Travel	*4,000 €*	*Yearly visits of project meetings and national/international conferences*	
Other	*2,923 €*	*Shipment of infectious viruses or generated cell lines*	
Sum: Total Budget:	350,000€		
Institutional Overhead:	0%		
Sum: Requested Budget (50% BMBF-share for SME)	350,000€		

8.1. Note regarding potential overlap with DZIF

This group is not a member of DZIF.

Project No.	**P3**
Title	Discovery of novel cellular barriers in zoonotic respiratory viruses at the protein level
Principal Investigator	*PD Dr. rer. nat. Albrecht von Brunn, Ludwig-Maximilians-University Munich*
Contribution to the One Health approach	Viral virulence and pathogenesis strongly depend on host factors. The identification of common cellular pathways used by virus families and diverse viruses in animal and humans will serve as predictors of transmissibility and pathogenesis of pre-pandemic viruses.
Abstract	**Intention and benefit for the consortium** Zoonotic respiratory viruses like MERS-CoV can cross species barriers and replicate in different hosts with no or little pathogenicity in animal reservoirs, but causing serious illness in humans. To understand barriers and pathogenicity it is important to gain knowledge on the function of the individual viral proteins in animal and human host cells, on their interactions with viral and cellular proteins and on the consequences of these interactions on cellular signaling pathways. Great knowledge has recently been accumulated on the interplay of cellular and proteins of various viral species, allowing the prediction of cellular pathways required for viral replication in general. We will utilize this knowledge to construct a new, quickly deployable Yeast-2-Hybrid (Y2H) screen, based on a core human cDNA library reduced to most important cellular signaling pathways involved in viral replication. Together with project **P2**, this resource will provide systems biology insight early during emerging virus outbreaks. **Technical highlights, novelty, uniqueness** We expect to generate a quickly applicable Y2H library of human cDNA expression clones with predictive potential regarding barriers, pathogenesis and prevention at the protein level. **Expected outcomes and perspectives** We will provide an “emergency” Y2H screening system for pre-pandemic viruses with proof of concept for MERS-CoV.

1 Working hypothesis and Research question(s)

Applying unbiased high-throughput yeast two hybrid (HTY2H) screening to the SARS-CoV orfeome with human cDNA libraries we have discovered cellular cyclophilin A as a prerequisite for Coronavirus (CoV) replication and inhibitors thereof as broadly acting antivirals. This outcome of the former SARS consortium is now pursued for in a drug repurposing/lead optimization project in the context of Deutsches Zentrum für Infektionsforschung (DZIF), without overlaps with the present proposal.

Main goals in the present proposal are i) the identification of virus-host protein interactions for a potential novel virus, with relevance for other emerging viruses (proof of concept for MERS-CoV) ii) the provision of criteria for viral virulence and epidemic risks based on molecular interactions and iii) the provision of rapidly deployable Y2H screening based on specific cellular checkpoints required for viral replication. This systems biology approach complements the consortium´s search for risk assessments of pre-pandemic agents by identification of cellular barrier markers at the protein level. The project is highly synergistic with project **P2**.

Methodological background: Proteomic changes have been studied for different viruses at the level of individual virus–host protein-protein interactions (PPI), organelles, and whole cells by techniques like 2D-SDS-PAGE and MassSpec (1). Major limitations of these approaches include lack of reproducibility and high throughput (HT) capabilities. Unprecedented molecular

information has been acquired from systems-level genome-scale RNA interference screens, as well as global affinity purification-mass spectrometry (2). Although these methods are very powerful, gene and protein expression are not the same and proteins and their post-translational modifications pose main factors in cellular and viral life cycle.
To fill these gaps we chose to analyze virus-host PPIs using HTY2H methods. We will develop our recent approaches of unbiased PPI screening of viral ORFs against human cDNA libraries further by constructing host factor Y2H libraries (HFY2HLIB) representing checkpoint genes for a number of important pathways, e.g. type I interferon and IFNγ -mediated signaling, ER stress response, Endosomal Complex Required for Transport (ESCRT) machinery, membrane traffic, ubiquitinases, kinases, chaperones etc. Genes will be selected on the basis of our own recent findings on SARS-CoV and HCoV-NL63 interactomes, literature and proteomic/RNA interference databases, e.g. Virhostome, FLUDB, VIPR etc. To test the usefulness of such host libraries we will use variants of MERS-CoV, the most recent zoonotic and pre-pandemic respiratory virus, as model organisms. Mutual incorporation of results of the RNAi/CRISPR/Cas-based systems project **P2** will be essential. The advancement over using the undirected cDNA library screening approach is the save of time and costs since positive hits do not have to be PCR-amplified, sequenced and blasted against gene banks in order to identify the host target. An optimistic, but reasonable goal is the quick (within a few months) identification of relevant antiviral targets on basis of this focused library of common cellular factors.
We envision to apply focused Y2H screening in any event of emergence of a novel pre-pandemic respiratory virus.

2. Own previous work and publications

We were one of the first groups applying HTY2H systems biology technologies to the study of PPIs of CoVs. As member of the former SARS consortium we have established protein interactomes of the highly pathogenic SARS-CoV and the mildly pathogenic HCoV-NL63 by screening interactions of all individual viral proteins against human cDNA libraries. This led to the identification of important cellular pathways and targets for the interference with CoV replication. Three prominent examples are the identification of a) the influence of nsp1 on the major immunity-related NFAT pathway, b) the cellular chaperon cyclophilin A (CypA) as a prerequisite for CoV replication with corresponding inhibitors [CsA, Alisporivir, NIM811] and c) tumor suppressor gene p53 as down-regulator of SARS-CoV replication via the interaction of the E3 ubiquitin ligase with the SARS unique domain (SUD) and viral PLpro (Ma-Lauer et al., under review). Developing human Y2H expression libraries containing defined genes of cellular pathways important for viral replication and barrier restriction does not repeat the “old” non-directional PPI screens with a new virus. It rather constitutes a consequent extension of our findings since we utilize the functional knowledge gained on genes identified by us and by others. Holding such a defined library at hand analysis of molecular barriers at the protein level will be much faster, cheaper and more efficient for **predicting molecular weaknesses of new agents.**

2.3. List of publications **(*=outcome from BMBF SARS consortium)**

*1. Carbajo-Lozoya, J., Y. Ma-Lauer, M. Malesevic, M. Theuerkorn, V. Kahlert, E. Prell, B. von Brunn, D. Muth, T. F. Baumert, C. Drosten, G. Fischer, and A. von Brunn. 2014. Human coronavirus NL63 replication is cyclophilin A-dependent and inhibited by non-immunosuppressive cyclosporine A-derivatives including Alisporivir. Virus Research 184:44-53.
*2. Carbajo-Lozoya, J., M. A. Muller, S. Kallies, V. Thiel, C. Drosten, and A. von Brunn. 2012. Replication of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E is inhibited by the drug FK506. Virus Research 165:112-117.
*3. Pfefferle, S., J. Schopf, M. Kogl, C. C. Friedel, M. A. Muller, J. Carbajo-Lozoya, T. Stellberger, E. von Dall'Armi, P. Herzog, S. Kallies, D. Niemeyer, V. Ditt, T. Kuri, R. Zust, K. Pumpor, R. Hilgenfeld, F. Schwarz, R. Zimmer, I. Steffen, F. Weber, V. Thiel, G. Herrler, H. J. Thiel, C. Schwegmann-Wessels, S. Pohlmann, J. Haas, C. Drosten, and A. von Brunn. 2011. The SARS-coronavirus-host interactome: identification of cyclophilins as target for pan-coronavirus inhibitors. PLoS Pathog 7:e1002331.
*4. von Brunn, A., S. Ciesek, B. von Brunn, and J. Carbajo-Lozoya. 2015. Genetic deficiency and polymorphisms of cyclophilin A reveal its essential role for Human Coronavirus 229E replication. Curr Op Virol 14:56-61.
*5. von Brunn, A., C. Teepe, J. C. Simpson, R. Pepperkok, C. C. Friedel, R. Zimmer, R. Roberts, R. Baric, and J. Haas. 2007. Analysis of intraviral protein-protein interactions of the SARS coronavirus ORFeome. PLoS.One. 2:e459.

Patents: - LMU Patent: COMPOSITIONS AND METHODS FOR TREATING CORONAVIRUS INFECTION, US Patent Publication No.US-20 16-008207 4-A 1. March-24-2016. Applicant: LMU Munich. Inventors: Nikolai Naoumov (Novartis) and Albrecht von Brunn (LMU)
- Internationale Patentanmeldung PCT/EP2015/000543 LMU Munich, „Treatment for Infection with a Coronavirus". Applicant: LMU München. Inventor: Nikolai Naoumov (Novartis) and Albrecht von Brunn (LMU)

3 Work plan including milestones

3.1. Aims

We aim to set up defined Y2H expression libraries for PPI analysis of viral ORFeomes with human genes reflecting cellular restriction factors and pathways essential for replication of diverse viruses. MERS-CoV will serve as a model pathogen. The setup should be applicable to any upcoming virus with identified genome composition.

3.2. Work planning

3.2.1. Step 1 – Host-Factor-Y2H-Library (HFY2HLIB)

3.2.1.1. Description of work

Many cellular genes involved in pathways important for viral replication are known. Information will be extracted from literature and virus-host interaction databases (Virhostome, FLUDB, VIPR etc.) of (mainly) positive strand RNA viruses. These include our own analyses on CoVs [(3), NL63 unpublished], influenza virus (2), Flaviviruses (4), kinome-based anti-SARS-CoV (5) and an ISG library (containing more than 350 human ISGs) screen of 14 viruses (6). All clonings will be performed in a GATEWAY-compatible manner. In close collaboration with **P5** we will start with a set of 20 classic antiviral ISGs of humans (MxA, MxB, GBP1, IFITM1, IFITM2, IFITM3, Viperin, ISG20, PKR, ISG15, tetherin, APOBEC3G, ADAR1, ISG56, ISG54, ZAPL, ZAPS, OAS1, OAS2, OAS3). A significant number of theses clones will be extracted from a library of about 12,000 human cDNAs clones. If it is necessary genes will be cloned from cellular mRNAs by reverse transcription and PCR. In a number of cases it will also be required to clone several isoforms of a gene as these may exhibit diverse functions. Y2H expression clones will be organized according to function in 96 well format as bait and prey libraries. 10 plates will contain ~950 genes plus controls. This number is reasonable to handle. Multiple copies will be stored at -80^{0}C. In parallel we will attempt to freeze-dry yeast cells within the plates for long-time storage and distribution to labs in case of new outbreaks.

3.2.1.2. Expected results

This sub-project will provide Y2H cDNA expression libraries of host genes involved in viral replication, antiviral response and pathogenicity. These will be applicable to study cellular barriers and targets for antiviral intervention of known and future emerging viruses. The clones will be made available to all consortium projects (**P1, P2, P4-P8**).

3.2.1.3. Milestones and contingency strategy

M1: Theoretical design and practical construction of various feature libraries (containing defined checkpoint genes) representing pathways like innate and adaptive immunity, ER stress response, membrane traffic, ubiquitinases, kinases, chaperones etc. (HFY2HLib). GATEWAY-compatible cloning of the various genes and where appropriate respective isoforms into pDONR entry and pDEST exression vectors (pGBKT7, pGADT7): month 24
M2: Evaluation of freeze-drying Y2H expression clones: month 6
Failure of this part is highly unlikely. Information on genes is publically available. Clones will be derived from our own cDNA (12,000 clones) library, from published libraries of colleagues and by RT-PCR and cDNA cloning of mRNA. All genes will be sequenced. If a desired gene cannot be received closely related family member genes will be used.

3.2.2. Step 2 – MERS-CoV ORFeome Y2H library construction
3.2.2.1. Description of work
As prototypes the cloned and error-corrected MERS-CoV EMC as well as the Korean isolate, provided by **P1**, will be used. 11 reading frames with 25 ORFs (16 Nsps + 9 structural/accessory) and subdomains will be PCR-amplified from BAC plasmids and cloned into GATEWAY-compatible pENTER plasmids. Subsequently they will be transferred into yeast expression vectors pGBKT7 (bait library) and pGADT7 (prey library) in a permutated (N- and C-terminal ORF fusion to DNA-binding and – activating domains) fashion. ORFs will also be cloned into mammalian expression vectors for confirmation of Y2H-positive hits and functional assays. We have available a battery of mammalian expression vectors with permutated tags (e.g. HA, c-myc, HIS, split YFP, GFP, RFP, protein A, Renilla).

3.2.2.2. Expected results
A library of all ORFs and subdomains thereof of two MERS isolates will be obtained. Due to GATEWAY compatibility, they can easily be transferred into yeast, mammalian and prokaryotic expression vectors as needed by all consortium projects (**P1, P2, P4-P8**).

3.2.2.3. Milestones and contingency considerations
M3: Design and practical construction of MERS-CoV (EMC) ORF Y2H library: month 6
M4: Design and practical construction of MERS-CoV (Korea) ORF Y2H library: month 6
Based on our recent experience on SARS-CoV and NL63 the MERS ORFeome cloning is straight forward with respect to design, selection of subdomains and cloning. For Y2H expression transmembrane regions will be omitted in order to avoid toxic effects. We do not expect necessary deviations from this strategy.

3.2.3. Step 3 – Y2H screening of MERS-CoV ORF Y2H libraries against human HFY2HLIB
The HFY2HLIB prey library will be arrayed on 96 well plates and individually screened with the MERS-CoV bait ORFs. Tests will be carried out in quadruplicates. Positive hits will be validated by our various PPI assays in mammalian cells. Interactomes will be analyzed bioinformatically.

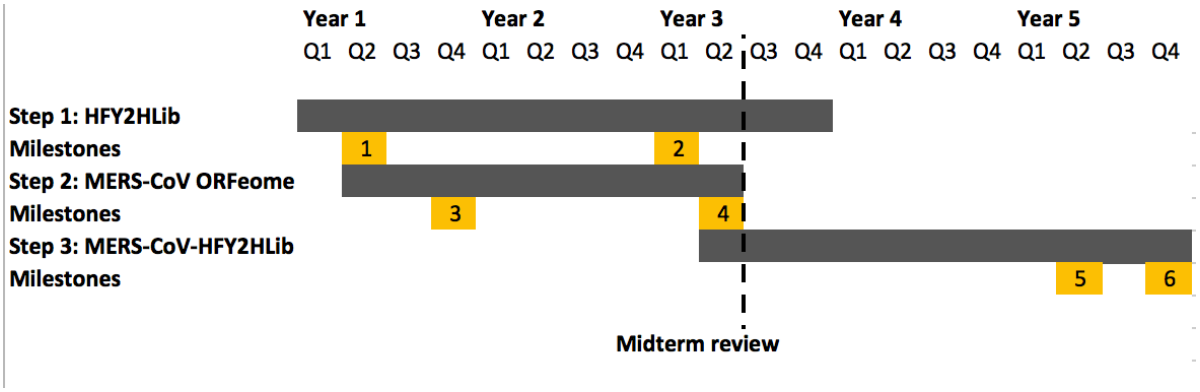
3.2.3.2. Expected results
As the human genes were preselected according to their supportive or abortive function for the replication of different viruses we expect positive Y2H hits in the low hundreds. These will be confirmed by mammalian PPI assays and evaluated with respect to their predictive value of pathogenicity and anti-viral potential (known and potential new inhibitors).

3.2.3.3. Milestones and contingency considerations
M5: Y2H screening of MERS-CoV ORF Y2H library against human HFY2HLIB and validation of Y2H-positive hits with mammalian protein-protein interaction assays: month 12
M6: Bioinformatic validation of cellular genes as prediction factors for pathogenesis and viral restriction: month: 6
We will not wait with Y2H screening until the final construction and cloning of the HFY2HLIB. I.e. as soon as we have “filled” a 96 well plate with human genes we will start screening with MERS-CoV ORFs ready to go.

3.3. Overview of milestones and time planning

3.3.1. Criteria for mid-term evaluation
Selection/cloning of cellular/viral Y2H expression clones will be done on a constant basis. The viral nucleic acid material is available *in toto* from the beginning on. The cellular genes will be collected step-by-step and the respective library is expected to be finalized towards the end of the first term. Y2H screens will start as soon as pathway families are cloned.

3.3.2. Gantt chart and milestones



Milestone 1: Construction of HFY2HLIB
Milestone 2: Evaluation of freeze-drying Y2H expression clones
Milestone 3: Construction of MERS-CoV (EMC) ORF Y2H library
Milestone 4: Construction of MERS-CoV (Korea) ORF Y2H library
Milestone 5: Y2H screening of MERS-CoV ORF Y2H libraries against human HFY2HLIB
Milestone 6: Bioinformatic validation of cellular genes as prediction factors

3.4. Considerations regarding dual use of research outcomes

The experiments of the project are designed to attain knowledge on the interplay of viral with host proteins and not to gain new viral functions. There is no DURC aspect.

4 Contribution to the consortium

4.1. Collaboration within the consortium

Acquiring preparedness to current and future outbreaks requires broad and systematic scientific work only achievable within a consortium. The consortium provides the ideal environment for reaching these goals. Successful long-term collaborations with members of the former SARS consortium provide an extremely fruitful basis for the envisaged synergies of the integrative MERS project. **P1** provides essential virus materials (MERS-CoVs/-cDNAs, BAC clones and newly emerging viruses) and the opportunity to test the functional significance of host factors and viral mutants at biosafety-level 3. The exchange of data and materials between our PPI with **P2** siRNA systems approaches of defining cellular barriers against CoVs are inevitable. **P3** provides a library of about 12,000 human cDNAs clones to other consortium members. The long-term experience of **P5** on innate immunity will help to decide on the choice of respective genes into our expression libraries. **P4** and **P5** will include our novel findings into their cellular assay systems and contribute them to the risk assessment platform in the second stage of the project. **P6** and **P7** will integrate results of **P2** and **P3** into its mucosal and human lung infection models, respectively. Results of the camel immunization project (**P8-P10)** on host immune factors (innate, adaptive) will be integrated into our Y2H expression libraries.

5 Quality assurance, standardization, data sharing

Materials, tools and data will constantly be exchanged and compared across the consortium.

6 Ethical and legal considerations

Animal experiments testing broad-spectrum antivirals will only be performed after approval by ethics committee.

7 Key references

1. Ma-Lauer Y, Lei J, Hilgenfeld R, & von Brunn A (2012) Virus-host interactomes--antiviral drug discovery. Current opinion in virology 2(5):614-621.

2. Tripathi S, et al. (2015) Meta- and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell Host & Microbe 18(6):723-735.
3. Pfefferle S, et al. (2011) The SARS-coronavirus-host interactome: identification of cyclophilins as target for pan-coronavirus inhibitors. PLoS Pathog 7(10):e1002331.
4. Le Breton M, et al. (2011) Flavivirus NS3 and NS5 proteins interaction network: a high-throughput yeast two-hybrid screen. BMC Microbiol 11:234.
5. de Wilde AH, et al. (2015) A Kinome-Wide Small Interfering RNA Screen Identifies Proviral and Antiviral Host Factors in Severe Acute Respiratory Syndrome Coronavirus Replication, Including Double-Stranded RNA-Activated Protein Kinase and Early Secretory Pathway Proteins. J Virol 89(16):8318-8333.
6. Schoggins JW, et al. (2014) Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. Nature 505(7485):691-695.

8 Financial summary (in Euros)

Personnel for 3.5 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student, E13, 65%	*146,675 €*	*42*	*Experimental work as described in chapters 3.2.1, 3.2.2. and 3.2.3..*
TA, E9, 50%	*48,000*	*24*	
Other resources for 5 years			
Type	Total Budget	Specification / Justification	
Consumables (42 month)	*81,564 €*	*per year in total:* *Molecular biology reagents (restriction/cloning enzymes, sequencing, antibodies, kits etc.:* *Cell culture, yeast, bacterial media;* *Plastic material for cultures:* *Antibiotics and other special reagents:*	*23,304 €* *12,000 €* *5,000 €* *3,304 €* *3,000 €*
Animal costs	-		
Equipment	-		
Travel	*12,000 €*	*Conferences, lab visits to other groups, consortium meetings*	
Other	*3,011 €*	*Shipments of infectious viruses from C. Drosten´s lab*	
Sum: Total Budget:	291,250 €		
Institutional Overhead:	20%		
Sum: Requested Budget (50% BMBF-share for SME)	349,500 €		

8.1. Note concerning potential overlaps with DZIF

Based on the outcome of the former BMBF SARS consortium we are now involved in the project part of DZIF (no infrastructural funding) in a drug repurposing/lead optimization project. The project is part of the DZIF TTU Emerging Infections. As our work is entirely dedicated to the testing and optimization of anti-Coronavirus lead substances and does not involve any further work to discover novel virus-host interactions, there is no overlap with the here-proposed project. The funding we receive through DZIF involves a PhD student position for 3 years. There is no possibility to use the DZIF resources for any work going beyond the work plan of the DZIF project.

Project No.	4.
Title	Efficiency of proteolytic activation of respiratory viruses as predictor for pandemic risk
Principal Investigator	*Prof. Dr. Stefan Pöhlmann, Deutsches Primatenzentrum (DPZ)*
Contribution to the One Health approach	We will exploit host cell factor usage as a predictor for viral transmissibility in and between reservoir hosts and humans.
Abstract	**Intention and benefit for the consortium**. Respiratory viruses at a pre-pandemic stage pose a severe threat to public health. The choice of cellular factors subverted by these viruses for spread can predict transmissibility and pathogenicity. We have identified the cellular serine protease TMPRSS2 as an activator of MERS-CoV, influenza A viruses and other respiratory pathogens and provided evidence that this protease is essential for viral spread and pathogenesis. Here, we will evaluate the efficiency of TMPRSS2 usage as a predictor of viral transmissibility and pathogenicity. For this, we will compare activation of MERS-CoV variants in novel cell systems and correlate the results with efficiency and TMPRSS2-dependence of viral spread in cultured respiratory epithelium and with viral pathogenicity. Pathogenicity will be determined in a non-human primate (NHP) model for lethal MERS-CoV infection. NHP resources are unique to this project and integral to the consortiums efforts to validate cell-based assays as predictors for pandemic risk. Collectively, our approach will equip the consortium with valuable *in vitro* and *in vivo* tools for rapid and accurate prediction of threats associated with emerging MERS-CoV variants and potentially other respiratory agents. **Technical highlights, novelty, uniqueness**. We will use our vast collection of TMPRSS2 reagents and unique access to NHP technology to generate novel, versatile assay systems to analyze the efficiency of viral activation by TMPRSS2 and its impact on viral biology. Conceptually, a correlation between efficiency of activation by TMPRSS2, transmissibility and pathogenicity would establish a novel paradigm in the coronavirus field with implications for prediction of pandemic potential and antiviral intervention. **Expected outcomes and perspectives**. We expect to establish an experimental workflow useful for predicting the pandemic risk of MERS-CoV variants and potentially other respiratory viruses activated by TMPRSS2.

1 Working hypothesis and Research questions

The cellular serine protease TMPRSS2 activates the glycoproteins of MERS-CoV, SARS-CoV and influenza A viruses (FLUAV) by cleavage and several lines of evidence suggest that TMPRSS2 activity is essential for full viral spread and pathogenicity in the host. We hypothesize that the efficiency of activation by TMPRSS2 is a predictor of viral inter- and intraspecies transmissibility and pathogenicity. We will investigate this hypothesis in the context of MERS-CoV, addressing the following questions: 1) Can quantitative and qualitative differences in TMPRSS2 usage by MERS-CoV isolates be revealed in cell culture systems, and can viral sequences be defined that are associated with efficient TMPRSS2 usage? 2) Do differences in TMPRSS2 usage correlate with the efficiency and TMPRSS2-dependence of viral spread in cultured respiratory epithelium and with viral amplification and pathogenicity in the host?

1.1. State of the art

MERS-CoV and other emerging viruses present in animal reservoirs and occasionally transmitted to humans but yet unable to sustain human-to-human transmission threaten public health (1). The rapid and accurate determination of the severity of this threat is a major challenge for researchers and public health authorities. This challenge will be met by the MERS consortium. An integral component of the consortiums approach is the characterization of host cell factors hijacked by emerging respiratory viruses for spread, since adaptation to efficient use of these factors may indicate high transmissibility and pathogenicity. Our previous work critically contributed to the identification of a host cell protein indispensable for spread of several respiratory viruses, the protease TMPRSS2.

TMPRSS2 cleaves coronavirus (CoV) spike proteins (S) and FLUAV hemagglutinin (HA) (2). Cleavage allows these proteins to transit into an active form and is indispensable for viral infectivity. However, endosomal cysteine proteases and furin were also reported to activate S-proteins and HA *in vitro* (3,4), raising the question which protease(s) facilitate(s) viral spread in the host. Studies employing knock-out mice demonstrated that diverse FLUAV critically depend on TMPRSS2 for HA activation, spread and pathogenesis (5), and polymorphisms in TMPRSS2 were found to be associated with influenza severity in human patients. Similarly, a serine protease-inhibitor active against TMPRSS2 blocked SARS-CoV spread and pathogenesis in a rodent model while a cysteine protease inhibitor had no effect (6). These results indicate that TMPRSS2 is a host factor critical for spread of emerging respiratory coronaviruses, FLUAV and likely other viruses, since recent studies revealed that also CoV 229E, human metapneumovirus, respiratory parainfluenza viruses and hepatitis C virus are activated by TMPRSS2. Moreover, these findings show that although several proteolytic pathways can be available for viral activation in cell culture, viruses may depend on single enzymes for activation in the host.

It is currently unknown whether the efficiency of TMPRSS2-mediated activation differs between viruses and whether these differences are associated with transmissibility and pathogenicity. Such an association is likely, since low expression of TMPRSS2 in certain parts of the aerodigestive tract might force viruses to optimize TMPRSS2 usage for robust spread and eventually human-to human transmission. We will investigate this scenario in the present project in the context of MERS-CoV infection.

1.2. Approach

Cell cultures can be used as tools to assess the potential of emerging viruses to enter and spread in the human population. Moreover, non-human primate (NHP) studies are central to risk assessment, since NHP are genetically most closely related to humans and frequently develop comparable disease after infection with human pathogens. Both components, novel cell culture systems and an NHP model, are integral to our approach to risk assessment in the context of MERS. Our study is based on the concept that MERS-CoV adaption to efficient usage of the host factor TMPRSS2 should predict viral transmissibility and pathogenicity. This concept is likely also applicable to other emerging viruses, since several viral pathogens hijack TMPRSS2 for spread.

To investigate our hypothesis, we will first generate assay systems, which allow us to quantify the efficiency of S-protein activation by TMPRSS2. Recombinant TMPRSS2 and cell lines expressing escalating amounts of TMPRSS2 proteins will be integral to our endeavors. Activation of MERS-CoV from well-studied outbreaks will be analyzed and S-protein sequences controlling TMPRSS2 usage will be defined. Next, we will assess whether viruses differing only in TMPRSS2 usage exhibit different capacities to replicate in respiratory epithelium, a prerequisite to efficient transmission, and to cause disease. For this, we will employ precision cut lung slices (PCLS) from NHP and a marmoset model of lethal MERS-CoV infection (7). A correlative analysis of the data will then reveal potential associations between activation efficiency, viral spread in respiratory epithelium and pathogenicity and will thus define the suitability of TMPRSS2 usage as a predictor for MERS-CoV transmissibility.

2 Own previous work and publications

2.1. Preliminary work.
We identified TMPRSS2 as an activator of SARS-CoV (1), MERS-CoV (2) and showed that this protease is expressed in viral target cells in the human lung (3) and demonstrated that usage of TMPRSS2/serine proteases is essential for FLUAV (4) and CoV (5) spread in rodent models. Studies on CoV activation by TMPRSS2 were conducted in the BMBF funded consortium SARS consortium while studies on FLUAV were funded by DFG. The proposed study builds on our previous work but addresses a new scientific question.

2.2. List of 5 publications

1) Glowacka I,…S. Pöhlmann. Evidence that TMPRSS2 activates the SARS-coronavirus spike-protein for membrane fusion and reduces viral control by the humoral immune response.
*J Virol. 2011 85:4122-34
2) Gierer S.,…S. Pöhlmann. The spike-protein of the emerging betacoronavirus EMC uses a novel coronavirus receptor for entry, can be activated by TMPRSS2 and is targeted by neutralizing antibodies.
*J Virol. 2013 87(10):5502-11
3) Bertram S,…Pöhlmann S, Soilleux EJ. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts.
PLoS One. 2012;7(4):e35876.
4) Hatesuer B,…Pöhlmann S, Schughart K. Tmprss2 is essential for influenza H1N1 virus pathogenesis in mice.
PLoS Pathog. 2013 Dec;9(12):e1003774
5) Zhou Y,...Pöhlmann (8th of 11), … Simmons G. Protease inhibitors targeting coronavirus and filovirus entry. Antiviral Res. 2015 Apr;116:76-84.

*=outcome from BMBF SARS consortium

3 Work plan including milestones

3.1. Aims
The goal of our work is to establish the usage of a host cell factor, TMPRSS2, as a molecular signature for transmissibility and pathogenicity of MERS-CoV.

3.2. Work planning

3.2.1. Step 1 – Characterization of TMPRSS2 usage by MERS-CoV isolates

3.2.1.1. Description of work
MERS-CoV isolates employ TMPRSS2 for S-protein activation. Our goal is to reveal potential isolate-specific differences in activation efficiency. A focus will be on the 2015 Korea outbreak strain, reservoir-borne viruses (generated by **P1**) and previously documented viral variants with exchanges near the S1/S2 border and the fusion peptide (i.e. potential cleavage sites). Moreover, viruses will be analyzed that were adapted to exclusive usage of TMPRSS2 or alternative activators by passaging in cell cultures. We will first investigate whether the S-proteins of the respective viruses differentially colocalize and interact with TMPRSS2, as determined by immunoprecipitation and FACS-based FRET assay. Next, we will test whether S-proteins presented on pseudoviruses require different concentrations of recombinant produced TMPRSS2 protease domain for activation. In parallel, we will engineer cells to express TMPRSS2 of human, camel and bat origin under control of an inducible promoter and test concentration-dependence of TMPRSS2-dependent activation of S-bearing pseudotypes (alternative activation pathways will be blocked). Interesting results will be confirmed by analysis of activation of authentic MERS-CoV, employing plaque assay, and mutagenic analysis of viruses with differential TMPRSS2 usage will reveal sequences controlling cleavability by TMPRSS2. Finally, it will be determined, jointly with **P5**, whether efficiency of activation by TMPRSS2 or choice of activating protease in general correlates with sensitivity towards interferon-induced antiviral effectors, which could explain why TMPRSS2 usage is critical in the host although alternative activation pathways are operative.

3.2.1.2. Expected results

We expect to reveal differences between MERS-CoV isolates in the efficiency of activation by TMPRSS2 and to identify the S protein sequences determining activation efficiency.

3.2.1.3. Milestones and contingency strategy
M1: *Cell systems suited to analyze TMPRSS2 usage*. In case stable cell lines cannot be generated, transient systems will be used. If purification of active TMPRSS2 protease domain is not feasible, trypsin will be employed as surrogate.
M2: *Identification of MERS-S sequences associated with TMPRSS2 usage*. In case analysis of passaged viruses does not provide insights into sequences determining TMPRSS2 usage, mutagenic analysis of computer predicted protease motifs in MERS-S will be employed.

3.2.2. Step 2 – Spread of MERS-CoV isolates in primary respiratory epithelium

3.2.2.1. Description of work
We will assess whether differential usage of TMPRSS2, as revealed within step 1, translates into differential replication of MERS-CoV isolates in primary respiratory epithelium. Moreover, we will investigate whether certain MERS-CoV isolates can employ TMPRSS2 jointly with related enzymes for activation, which may broaden the viral cell tropism. For this, we will employ PCLS from marmosets. We have previously analyzed FLUAV infection of marmoset PCLS jointly with collaborators at DPZ and will now establish the entire workflow in the laboratory, including detailed characterization and standardization to ensure optimal reproducibility of results (i.e. marker expression, viability). Dependence on TMPRSS2 activity for viral spread will be assessed employing protease inhibitors and specific siRNAs delivered by liposome-based transfection reagents. This approach will also be used to determine whether host factors identified by **P2** and **P3** are required for viral spread. The target cells of viruses that are relatively insensitive to blockade of TMPRSS2 activity will be investigated employing immunohistochemistry with focus on the expression of TMPRSS2-related enzymes able to activate MERS-CoV in transfected cells. Finally, the viruses with the most interesting phenotypes will be further analyzed in cultures of primary human respiratory epithelium and these efforts will be made jointly with **P6** and **P7**.

3.2.2.2. Expected results
We expect to uncover whether efficiencies of TMPRSS2 usage and spread in respiratory epithelium correlate and whether certain MERS-CoV isolates can employ TMPRSS2-related enzymes for spread in respiratory target cells.

3.2.2.3. Milestones and contingency strategy
M3: *Protocols for generation and characterization of PCLS cultures.*
M4: *Analysis of the efficiency and TMPRSS2-dependence of viral spread in PCLS.* If siRNA delivery via transfection does not yield the expected results, a virus-like particle system for siRNA delivery available at DPZ can be adapted to use in marmoset PCLS.

3.2.3. Step 3 – Amplification and pathogenesis of MERS-CoV isolates in marmosets

(also refer to "Formblatt C" on animal experimentation, as attached)
3.2.3.1. Description of work
Viral amplification in tissues relevant for transmission and viral pathogenesis as determined in NHP models are important predictors of the threat imposed by an emerging virus. Analyzing these parameters is integral to the efforts of the consortium to predict the pandemic potential of MERS-CoV isolates. We will employ a published marmoset model (8) for lethal MERS-CoV infection to determine amplification and pathogenicity of MERS-CoV variants. A particular focus will be on the Korean outbreak strain, which is used by all projects for validation, and on viruses with differential activation by TMPRSS2 but other viruses identified as interesting by **P1**, **P6** and **P7** can also be analyzed. A detailed description of the planned studies is provided in Formblatt C, animal studies.

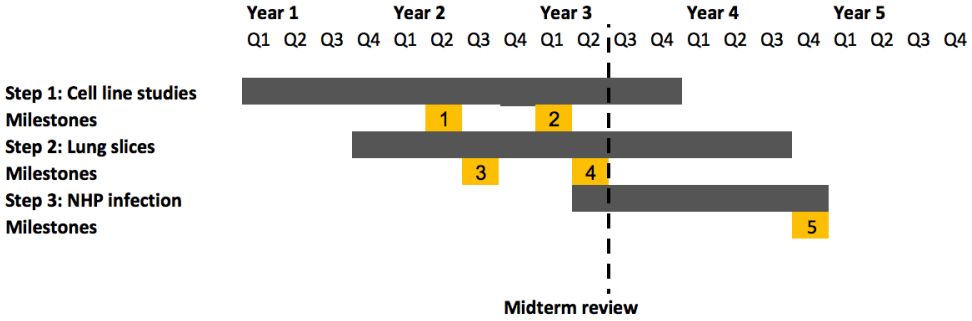
3.2.3.2. Expected results
We expect to determine whether efficiency of TMPRSS2 usage *in vitro* and spread and pathogenicity of MERS-CoV variants *in vivo* correlate.

3.2.3.3. Milestones and contingency strategy
M5: *Analysis of pathogenicity of MERS-CoV isolates in marmosets.* Variability in the efficiency of viral amplification and severity of disease, potentially due to differences in the genetic background, might require adjusting group sizes.

3.3. Overview of milestones and time planning

3.3.1. Criteria for mid-term evaluation
The establishment of cell lines suitable to determine the efficiency of TMPRSS2 usage, the identification of S-protein sequences controlling TMPRSS2 usage and establishment of PCLS cultures are central to the proposed project (M1, M2 and M3) and can serve as criteria for mid-term evaluation. Infection of NHP depends on completion of the refurbishing of the BSL3 animal facilities at DPZ and is thus planned in the second half of the project.

3.3.2. Gantt chart



3.4. Considerations regarding dual use of research outcomes
None of the planned studies will result in a gain of function.

4 Contribution to the consortium
This project proposes complex, technically challenging studies, which heavily rely on partners with expertise in diverse research areas. We particularly depend on P1 for viruses, P5 for analysis of IFN induced effectors and on P6, P7 for models of human respiratory epithelium. We will supply partners with PCLS and with a NHP model for lethal MERS-CoV infection. These resources are unique to this project and the NHP model is integral to the consortiums efforts to validate the workflow for risk assessment. We will collaborate with all partners within the areas infection phenotyping and risk assessment, as detailed below. In addition, our surrogate systems should help to quantify vaccine induced antibody responses.

Partner	Project area	Type of collaboration
P1, Drosten	Infection phenotyping	Viruses, functional studies
P2 + P3, von Brunn, Karlas	Infection phenotyping	New host cell factors
P5, Weber	Infection phenotyping	Protease use and IFN responses
P6, Thiel	Risk assessment	Infection of respiratory epithelium
P7, Hippenstiel and Wolff	Risk assessment	Infection of respiratory epithelium
P8, Volz and Sutter	Vaccination	Humoral immune responses

5 Quality assurance, standardization, data sharing
We will carefully characterize, control and validate our experimental systems and generate detailed protocols for our assays. All reagents and protocols will be provided to the consortium and health agencies for risk assessment.

6 Ethical and legal and considerations

The DPZ has long standing expertise and facilities for NHP studies. The appropriate permits for animal experimentation are either available or will be applied for.

7 Key references

1. Vijay R, Perlman S. Middle East respiratory syndrome and severe acute respiratory syndrome. Curr Opin Virol. 2016, 16:70-6.
2. Simmons G et al. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. Antiviral Res. 2013, 100(3):605-14
3. Simmons G et al. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci U S A. 2005, 102(33):11876-81.
4. Millet JK, Whittaker GR. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. Proc Natl Acad Sci U S A. 2014, 111(42):15214-9.
5. Tarnow et al. TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza A virus in mice. J Virol. 2014, 88(9):4744-51.
6. Zhou Y et al. Protease inhibitors targeting coronavirus and filovirus entry. Antiviral Res. 2015, 116:76-84.
7. Falzarano D et al. Infection with MERS-CoV causes lethal pneumonia in the common marmoset. PLoS Pathog. 2014 Aug 21;10(8):e1004250.

8 Financial summary (in Euros)

Personnel for 4 years

Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student, E13, 65%	*164,000 €*	*48*	*Experimental work as described in 3.2.1. and 3.2.2.*
Technician (E9)	*45,000 €*	*12*	*Experimental work as described in 3.2.3.*

Other resources for 4 years

Type	Total Budget	Specification / Justification
Consumables	*80,000 €*	*Plastic dishes, FCS, media, cytokines for cell culture* *Reagents for cloning and sequencing* *Reporter assays* *PCR reagents for quantification of viral genome copies* *Reagents for characterization of PCLS/infected NHP*
Animal costs	*55,000 €*	*Per diem (maintenance) for 24 animals, provision (purchase costs) will be covered by internal funds*
Equipment	-	
Travel	*7,500 €*	*Yearly visits of national and international conferences*
Other	*6,000 €*	*Shipments of infectious viruses from Bonn*

Sum: Total Budget:	357,500 €
Institutional Overhead:	20%
Sum: Requested Budget (50% BMBF-share for SME)	429,000 €

8.1. Note regarding overlap with DZIF

This institution/group is not a member of DZIF.

Formblatt C, animal studies, P4

Background and objectives:

The Middle East Respiratory Syndrome Coronavirus (MERS-CoV) causes severe and frequently fatal disease in human patients. However, it is currently impossible to predict transmissibility of MERS-CoV variants. The MERS consortium tackles this problem by developing an array of *in vitro* tools for risk assessment. For validation of these tools it is essential to determine whether MERS-CoV variants, for which cell culture systems predict altered transmissibility, are indeed differentially amplified in an animal model, which adequately mirrors MERS-CoV infection of humans. A marmoset model for lethal MERS-CoV infection has been previously documented (Falzarano *et al*, PLOS Pathogens, 2014, ref. 8) and will be employed for this purpose (jointly with the Infection Models Unit and Infection Pathology Unit of DPZ). Our first goal is to assess whether the MERS-CoV reference strain EMC and MERS-CoV variant D510G, which was detected in two Korean patients and is believed to be attenuated, are differentially amplified in experimentally inoculated marmosets and cause disease of different severity. One of the *in vitro* tools designed to assess MERS-CoV transmissibility are cell culture systems, which allow determining the efficiency of viral activation by the protease TMPRSS2. Therefore, the second goal of the animal studies is to investigate whether MERS-CoV variants, which differ only in spike protein sequences controlling TMPRSS2 usage, exhibit differential amplification and disease induction in marmosets.

The marmoset model will be used essentially as documented. In experimentally infected animals, the efficiency of viral amplification in nose, throat, trachea and, to a lesser degree, upper lung lobe can be considered as indicative of viral transmissibility. Viral genome copies present in these tissues will be measured at defined time points (nose, throat) or at end point (trachea, upper lung lobe), employing quantitative reverse transcription-PCR (qRT-PCR). Pathogenicity will be determined by assessing the clinical score twice daily and by analyzing lung pathology at end point, using immunohistochemistry and microscopic analysis. Similar information cannot be obtained in murine models of MERS-CoV infection, which are based on animals transgenic for human CD26 (the MERS-CoV receptor), due to differences in the structures of the human and the murine respiratory tract and due to altered expression levels and cell type specificity of CD26 in tissues of transgenic mice relative to non-human primates (NHP) and humans. Collectively, experimentally infected marmosets are currently the best model for lethal MERS-CoV infection of humans and will be employed to validate the tools for risk prediction developed by the MERS consortium.

***Methods**:*

a. Study design
Two experiments are planned, as outlined above. Each experiment will include one experimental and one control group, each containing 6 animals. Animals will be randomly distributed between groups. Clinical score will be determined by veterinarians not informed about the experimental design, viral load will be determined from anonymized samples.

b. Experimental procedures
The DPZ has ample experience in the experimental infection of non-human primates (NHP) with diverse viral pathogens, including studies with respiratory syncytial virus and monkeypox virus, both of which can be transmitted via the air. All animal work will follow relevant national and international guidelines. Good veterinary practice will be applied to all procedures whenever animals are handled. All measures will be performed according to the regulations of the German Welfare Act (Tierschutzgesetz der Bundesrepublik Deutschland) and the European Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. Work will commence only after approval by the governmental veterinary authority of Lower Saxony (Lower Saxony State Office for Consumer Protection and Food Safety, LAVES). For analysis of MERS-CoV amplification and pathogenesis, common marmosets (*Callithrix jacchus*)

will be anesthetized employing isoflurane and ketamine and then inoculated with MERS-CoV intranasally with 100 µl in each nare, 500 µl orally, 500 µl intratracheally and 50 µl in each eye with DMEM containing 4 x10^6 TCID50/ml (total dose 5.2 x10^6 $TCID_{50}$), as previously published. Subsequently, clinical score will be assessed twice daily, employing published parameters, and animals will be euthanized at a clinical score of 35 or higher. Nasal and throat swaps will be taken at days 1, 3, 5 and 6 for qRT-PCR analysis of viral genome copies. At day 6, at which highest clinical score was measured in a previous study, the animals will be scheduled for necropsy. At this point, lung pathology will be determined and pulmonary and extrapulmonary viral amplification will be assessed by qRT-PCR. The experiments are scheduled for years 3 and 4 of the project and will be conducted in the BSL3 animal facilities of DPZ. The animal facilities are designed for research with airborne BSL3 agents and contain three separate units for NHP experiments as well as a separate room for necropsy, containing adequate tables, lights and availability of narcotics. The BSL3 animal facilities are currently being refurbished and will be available in 2019.

c. Experimental animals
In keeping with published data, male common marmosets aged 2-6 years will be employed for the planned studies. The animals will be obtained from DPZ breeding colonies (F2 generation or higher).

d. Housing and husbandry
Cages for housing of individual animals will be used that comply with EU directive 2010/63. Cages of animals belonging to one group will be placed side-by-side to allow social interactions. Animals will be fed with pellets, fruits, vegetables, insects, corn and food cocktails specifically designed for marmosets. Food and water will be continuously available without restrictions, temperature, light and humidity will be adjusted to marmoset needs

e. *Sample size*
A total number of 12 animals will be used per experiment. For each experiment two groups of animals will be examined, each containing 6 animals. Animal numbers are based on statistical calculation, as stated below.

f. Allocating animals to experimental groups
The animals will be randomly assigned a number and distributed between the two groups.

g. Experimental outcomes
The primary outcomes are viral genome copies in nose, throat, lung and other organs as well as clinical score. Secondary outcome will be lung pathology, since this parameter cannot be as readily quantified as the aforementioned ones.

h. Statistical methods
The G*Power program (http://www.gpower.hhu.de/) was employed for statistical power analysis. On the basis of expected 10-fold differences in viral load and 4-fold differences in clinical score, respectively, between reference and test strain, significant differences between groups with n = 6 can be detected with a power of 0.8.

Project No.	5
Title	Innate immunity phenotype of viruses
Principal Investigator	Prof. Dr. rer. nat. Friedemann Weber and Prof. Dr. med. John Ziebuhr, University of Giessen
Contribution to the One Health approach	A key aspect of viral pathogenesis is the so-called innate immunity phenotype. This important virulence marker encompasses the capacity of viruses to avoid innate immune system activation, provoke inflammatory responses, and resist the antiviral action of interferons. We will generate tools for measuring the innate immunity phenotypes of prepandemic respiratory viruses in order to achieve a rapid, science-based risk assessment.
Abstract	**Intention and benefit for the consortium and for public health** Type I interferons (IFN-alpha/beta) and other cytokines are produced and secreted by virus-infected cells. IFNs trigger the expression of the so-called ISGs (IFN-stimulated genes) that can have direct antiviral activity. The ability of viruses to cope with these antiviral host responses ("innate immunity phenotype") is an important mechanism of virulence. Highly pathogenic viruses suppress the upregulation of IFN and other immediate-early genes, and counteract the antiviral action of specific ISGs. Viruses also upregulate proinflammatory responses, leading to tissue damage. Knowledge on the innate immunity phenotype of emerging MERS-CoV variants and other prepandemic respiratory viruses will provide robust data for risk assessment. **Technical highlights, novelty, uniqueness** Our project will develop novel tools and methods to rapidly assess the extent by which viruses dysregulate innate host responses and resist antiviral IFN action. Moreover we will establish systems that allow the identification of antiviral ISG products of humans that are capable to inhibit any given virus. **Expected outcomes and perspectives** We expect to generate tools that allow to rapidly measure the innate immunity phenotype of emerging viruses as a science-based predictor of pathogenicity.

1 Working hypothesis and Research question(s)

A main aim of our consortium is to set up a rapid risk assessment pipeline for viruses. The pipeline will be modeled for MERS-CoV, but once established it can be applied to any emerging virus. Our sub-project will deal with the interaction of pathogens with the innate immune barrier. IFNs and other cytokines are the first line of the antiviral host defense. IFNs trigger the expression of more than 300 ISGs, and several of them have documented antiviral activity (1). Pathogenic viruses provoke the induction of pro-inflammatory cytokines, but counteract the induction of IFN, block IFN signaling, or target individual ISG products. The quality and strength of cytokine induction, IFN evasion, and ISG sensitivity ("innate immunity phenotype") is an important marker of virulence.

We have previously observed that MERS-CoV has the ability to block the upregulation of IFNs and other cytokines (2), just like SARS-CoV (3). MERS-CoV, however, was found to be much more sensitive to IFN. This had led us to the risk assessment that MERS-CoV has not the same epidemical potential as SARS-CoV (2).

Among the hundreds of ISGs, about forty were characterized as being antiviral (1). Prominent examples are the IFITMs, Mx, viperin, ISG20, OAS/RNaseL, and PKR. Some of these inhibit a wide range of viruses, while others act rather virus-specific. Remarkably, little is known about ISGs against SARS-CoV or MERS-CoV (4). Moreover, for any prepandemic virus it is important

to know its innate immunity phenotype, as only then the specific “toughness” and Achilles’ heels will be known.
We will establish systems for rapid assessment of the cytokine (including IFNs) and antiviral gene induction profile and the specific IFN sensitivity profile of viruses in cell culture. Moreover, we will generate a library of cell lines that inducibly express individual ISGs. As final outcome, we envision 96-well plate-based, machine-readable assays for innate immunity induction profiles and IFN and ISG sensitivity. Such systems could provide rapid information on the innate immunity phenotype of viruses, thus serving the risk assessment pipeline of the consortium.

2 Own previous work and publications

We had shown that SARS-CoV and MERS-CoV prevent IFN and cytokine production in host cells, and that MERS-CoV is more IFN-sensitive than SARS-CoV (Spiegel et al., 2005; Zielecki et al., 2013). The blockade of IFN induction by SARS-CoV could be partially overturned by pretreating with small amounts of IFN (Kuri et al., 2009). In addition, we identified the ADRP domain of SARS-CoV as an anti-IFN mechanism (Kuri et al., 2011). We also established a small-scale ISG screen, and identified Viperin as an inhibitor of tick-borne encephalitis virus (TBEV) (Upadhyay et al., 2014). The ISG screening system proposed here will be a modern version, using rapid cloning and chromosome integration methods, as well as employing cells that are broadly infectable.

2.1. List of 5 publications

1. Spiegel M, Pichlmair A, Martínez-Sobrido L, Cros J, García-Sastre A, Haller O, Weber F. Inhibition of Beta interferon induction by severe acute respiratory syndrome coronavirus suggests a two-step model for activation of interferon regulatory factor 3.
J Virol. 2005; Feb;79(4):2079-86.

*2. Kuri, T., X. Zhang, M. Habjan, L. Martínez-Sobrido, A. García-Sastre, Z. Yuan, F. Weber. Interferon priming enables cells to partially overturn the SARS-Coronavirus-induced block in innate immune activation.
J. Gen. Virol. 2009; Nov;90(Pt 11):2686-94

*3. Kuri, T., K. K. Eriksson, A. Putics, R. Züst, E. J. Snijder, A. D. Davidson, S. G. Siddell, V. Thiel, J. Ziebuhr, F. Weber. The ADP-ribose-1”-monophosphatase domains of SARS-coronavirus and Human coronavirus 229E mediate resistance to antiviral interferon responses.
J. Gen. Virol. 2011; Aug;92(Pt 8):1899-905

*4. Zielecki, F., M. Weber, M. Eickmann, L. Spiegelberg, A. M. Zaki, M. Matrosovich, S. Becker, F. Weber. Human cell tropism and innate immune system interactions of human respiratory coronavirus EMC compared to SARS-coronavirus.
J. Virol 2013; May;87(9):5300-4

5. Upadhyay AS, Vonderstein K, Pichlmair A, Stehling O, Bennett KL, Dobler G, Guo JT, Superti-Furga G, Lill R, Överby AK, Weber F. Viperin is an iron-sulfur protein that inhibits genome synthesis of tick-borne encephalitis virus via radical SAM domain activity.
Cell Microbiol. 2014; Jun;16(6):834-48.

(*=outcome from BMBF SARS consortium)

3 Work plan including milestones

3.1. Aims

The final aim of our project is the rapid assessment of the cytokine/IFN induction and IFN sensitivity profile in human cells.

3.2. Work planning

3.2.1. Step 1 - Systems for rapidly measuring innate immunity induction

There are commercial real-time RT-PCR arrays that measure mRNAs for innate immunity (e.g. Antiviral Response RT^2 Profiler PCR Array, Qiagen), but these do not encompass all the cytokines and antiviral genes that in our experience are key to the understanding of antiviral host responses. We will set up our own, RNA virus-optimized real-time RT-PCR array, using genes that we either found ourselves (5, 6) or others (7) as being excellent markers of infection or inflammation (e.g. IP-10), or are suppressed by viruses. We will test the regulation of various cytokines, ISGs, and stress-related markers against a series of viruses of the *Coronaviridae*

(collaboration with **P1**), Influenza viruses, and *Paramyxoviridae* with different virulence. The progress of the project will show whether it will be necessary to separate the markers for plus- and minus strand RNA viruses, as these have very different replication and anti-IFN strategies. It is also important to measure at different time points after infection, e.g. 3, 8 and 16 h p.i.. As cells we will mostly use human A549 cells, but others (e.g. Caco-2, epithelial cells) can also be considered. After having collected the best innate immunity induction (or suppression) markers by real-time RT-PCR, we will have specific arrays to be set up by Qiagen.

3.2.1.2. Expected results
Real-time RT-PCR arrays to comprehensively measure the virus-provoked innate immunity induction profile as a predictor of virulence.

3.2.1.3. Milestones and contingency strategy
M1: Selection of real-time RT-PCR markers for innate immunity induction: month 12
M2: Real-time RT-PCR arrays to rapidly measure innate immunity induction: month 24

It might be possible (though unlikely), that the innate immune induction profiles of viruses are too heterogeneous for drawing robust conclusions. In that case we will separate the sets of markers according to the different virus groups (i.e. plus- vs minus-strand RNA viruses).

3.2.2. Step 2 – IFN/ISG sensitivity profile
3.2.2.1. Description of work
For measuring overall IFN sensitivity, cells will be pretreated with different amounts of IFNs, and virus replication assayed by real-time RT-PCR or conventional plaque assays.
For the specific ISG sensitivity profile, we will generate a large set of cell clones that inducibly express a library of ISGs. The primate CV-1 cell line is chosen since it is highly virus-permissive (8), in contrast to the HEK293s we had used for TBEV (9). ISG cDNAs will be produced and cloned into a tetracycline (Tet)-inducible expression plasmid. A commercial CV-1 Flp-In cell line is available (Thermo Fisher Scientific). It contains a chromosomal Flp recombination target (FRT) site, thus allowing stable insertion of cDNA expression cassettes containing FRT sites. To obtain inducible cDNA expression, we have to produce a Flp-In TR cell line by chromosomal integration of a Tet-repressor (TR) gene. This cell line will be the basis for integration of Tet-inducible ISG expression cassettes. We will start with classic antiviral ISGs (MxA, MxB, GBP1, IFITM1 to 3, Viperin, ISG20, PKR, ISG15, tetherin, APOBEC3G, ADAR1, ISG56, ISG54, ZAPL, ZAPS, OAS1 to 3), but the list of clones will be constantly growing over time. The tools and experiences of **P3** will be essential for rapid cloning by the GATEWAY system. New restriction factors or interactors discovered by **P2** or **P3** will also be integrated in our assays. Of note, we have decided to not use the constitutively expressing, lentiviral system of Schoggins et al., (1) to avoid any potential bias due to autocrine suppression of the lentiviral vector by particular ISGs, and to circumvent toxic effects of ISGs like e.g. PKR.
The reporter MERS-CoV strains of **P1** will serve to rapidly assess the suitability of the ISG clones. For other viruses used subsequently, real-time RT-PCRs, plaque assays, and direct cytopathic effects are readout for ISG action. Once a well-characterized set of ISG-expressing cell clones is available, we will attempt to seed an array of cell clones on 96 well plates, and freeze them down in a way they can be stored and used shortly after thawing.

3.2.2.2. Expected results
A set of ISG-expressing cell clones as a tool to determine IFN and ISG sensitivity of viruses, thus contributing to predict virulence with some likelihood.

3.2.2.3. Milestones and contingency strategy
M3: Tools and methods to test the IFN sensitivity of viruses: month 12
M4: Basic set of cell clones inducibly expressing human ISGs: month 30
Individual ISGs that may not be expressible. Also, it is possible the cell are not functional if frozen down in 96 well plates. These obstacles would not endanger the whole sub-project.

3.2.3. Step 3 – Innate immunity phenotypes of MERS-CoV strains and other respiratory viruses
3.2.3.1. Description of work
We will obtain profiles of innate immune induction and IFN/ISG sensitivity for the 30 MERS-CoV isolates and the mutants available from **P1**, and compare with SARS-CoV. Moreover, we will test our DZIF reference strain collection of respiratory viruses.

3.2.3.2. Expected results
Comprehensive data on innate immunity phenotypes of respiratory viruses, allowing to assess their correlation with virulence.

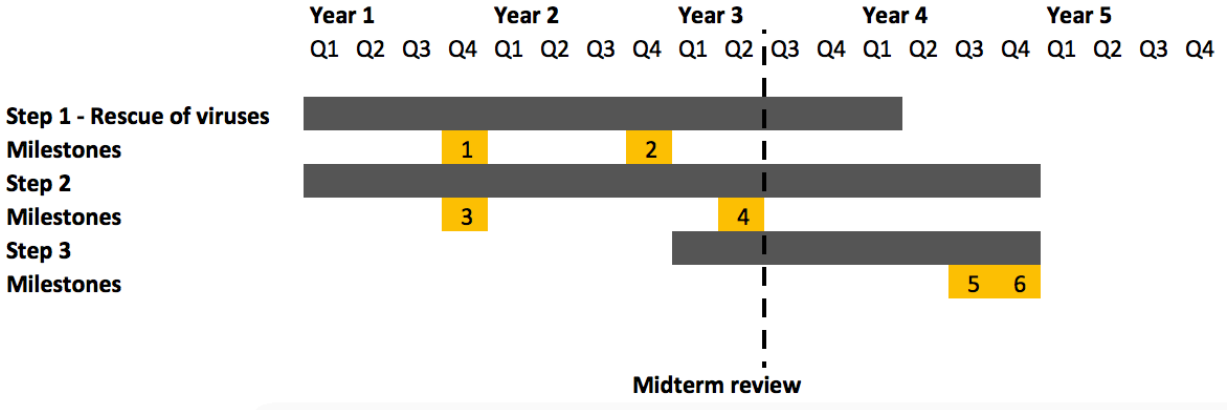
3.2.3.3. Milestones and contingency strategy
M5: Innate immunity induction by MERS-CoV strains, mutants, and other respiratory viruses: month 48
M6: IFN/ISG sensitivity profile of MERS-CoV strains and mutants compared to SARS-CoV: month 48
Given that Steps 1 and 2 will proceed as planned, we see no difficulties in achieving results.

3.3. Overview of milestones and time planning

3.3.1. Criteria for mid-term evaluation
The inclusion of innate immunity markers and ISGs will occur on a constant basis. As criteria for mid-term review we envision:

Milestone 1: A selection of marker genes to measure innate immunity induction
Milestone 2: Gene array with marker genes of innate immunity induction
Milestone 3: IFN sensitivity assay
Milestone 4: A set of CV-1 cell clones that inducibly express ISGs
Milestone 5: Innate immunity induction by MERS-CoV strains and other respiratory viruses
Milestone 6: IFN/ISG sensitivity profile of MERS-CoV strains and other respiratory viruses

3.3.2. Gantt chart



3.4. Considerations regarding dual use of research outcomes
As there are no experiments involved that lead to a gain of function, the project has is no DURC aspect

4 Contribution to the consortium
4.1. Reliance on work in the consortium
Such a broad and systematic approach is only possible within a consortium. **P1** possesses a huge and unique series of virus isolates and MERS-CoV mutants which are pivotal for our project. Moreover, this group is on the front line of virus outbreaks, ensuring rapid access to new viruses, and are experts in virus measurements. Additionally, our own diagnostic units for veterinary (F. Weber) and medical (J. Ziebuhr) viruses will be a rich source of virus isolates, allowing immediate phenotypical comparisons of any newly emerging virus with its close

relatives. We will constantly compare our data with those of **P6** and **P7** to gain insights in the robustness of the predictions and the assessment of virulence. For our ISG expression work we will strongly interact with the system biology projects **P2** and **P3**, and constantly share experiences with **P4**.

4.2. Collaborations within the consortium

Partner	Project area	Type of collaboration
Project 1, Muth, Drosten	Infection phenotyping	Provision of viral strains and mutants
Project 2, Karlas	Risk assessment	Antiviral restriction factor identification and testing
Project 3, von Brunn	Risk assessment	Antiviral restriction factor identification and testing. Expertise in rapid cloning
Project 4, Pöhlmann	Risk assessment	Molecular signatures for pathogenicity
Project 6, Thiel	Risk assessment	ISG selection, data comparison with ALI cell cultures
Project 7, Hoppe, Hippenstiel, Wolff	Risk assessment	Data comparison with human lung tissue model

5 Quality assurance, standardization, data sharing

We will constantly exchange and compare our data across the consortium, and share our tools and methods.

6 Ethical and legal and considerations

No animal or human experiments involved

7 Key references

1. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 472:481-U545.
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5. Kuri T, Zhang X, Habjan M, Martinez-Sobrido L, Garcia-Sastre A, Yuan Z, Weber F. 2009. Interferon priming enables cells to partially overturn the SARS coronavirus-induced block in innate immune activation. The Journal of general virology 90:2686-2694.
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7. Dixit E, Boulant S, Zhang Y, Lee AS, Odendall C, Shum B, Hacohen N, Chen ZJ, Whelan SP, Fransen M, Nibert ML, Superti-Furga G, Kagan JC. 2010. Peroxisomes are signaling platforms for antiviral innate immunity. Cell 141:668-681.
8. Hronovsky V, Plaisner V, Benda R. 1978. CV-1 monkey kidney cell line -- a highly susceptible substrate for diagnosis and study of arboviruses. Acta virologica 22:123-129.
9. Upadhyay AS, Vonderstein K, Pichlmair A, Stehling O, Bennett KL, Dobler G, Guo JT, Superti-Furga G, Lill R, Overby AK, Weber F. 2014. Viperin is an iron-sulfur protein that inhibits genome synthesis of tick-borne encephalitis virus via radical SAM domain activity. Cell Microbiol 16:834-848.

8 Financial summary (in Euros)

Personnel for 4 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student, E13, 65%	164,986 €	48	Establishing and validating assays as described in 3.2.1, 3.2.2. and 3.2.3.
Technician (E9/2)	180,000 €	48	Cloning, maintaining the library of cell clones, large-scale screens, testing of virus isolates in the assays of 3.2.1, 3.2.2. and 3.2.3.
Other resources for 4 years			
Type	Total Budget	Specification / Justification	
Consumables	88,000 €	Real-time RT-PCR reagents and kits, other molecular biology reagents (DNA oligos, transfection agents, DNA preparation, reporter assays, PCR, TA-cloning) cell culture media, cytokines, antibiotics, other special reagents, sterile plasticware for tissue culture, BSL3-specific protective clothing	
Animal costs	-		
Equipment	-		
Travel	7,500 €	Conferences, visits to other groups, consortium meetings	
Other	4,000 €	Shipments of infectious viruses from Christian Drostens lab	
Sum: Total Budget:	444.468 €		
Institutional Overhead:	20%		
Sum: Requested Budget (50% BMBF-share for SME)	533,362 €		

8.1. Note regarding overlap with DZIF

Gießen is a member of DZIF. The Institute of Virology in the veterinary medicine faculty (F. Weber) receives no funding through DZIF. F. Weber is the main recipient of the here-proposed grant. The Institute of Medical Virology (J. Ziebuhr) receives DZIF project funding (no infrastructure funding) for antiviral drug screening in the context of the DZIF TTU Emerging Infections (currently limited until 2018). While there is no overlap with the partial involvement of J. Ziebuhr in the present project, the DZIF funding has been essential to create the resource that J. Ziebuhr will contribute to the here-presented work.

Project No.	**6**
Title	Phenotypic characterization of emerging respiratory viruses in the primary airway epithelium
Principal Investigator	Prof. Dr. rer. nat. Volker Thiel, University of Bern Co-applicant: Dr. Ronald Dijkman
Contribution to the One Health approach	Zoonotic respiratory viruses will be analyzed in primary airway epithelial cultures derived from humans and reservoir animals in order to assess the risk of transmission and to develop efficacious strategies to combat respiratory virus infections in animals and humans.
Abstract	**Intention and benefit for the consortium** Host innate immune responses are of particular importance in the airway epithelium that represents the entry port of respiratory virus infection. By using primary human and animal airway epithelial cultures we will rapidly assess the interaction of emerging zoonotic respiratory viruses with host innate immune responses in these primary target cells. Specifically, we will provide biological signatures of host epithelial cell responses to infection of a panel of MERS-CoV isolates and mutants as well as to prototype respiratory viruses of selected virus families. The primary airway system will be refined towards an experimental infection platform that allows for rapid and robust risk assessment and we will make this technology available for the consortium. **Technical highlights, novelty, uniqueness** By using *transgenic* primary human and animal airway epithelial cultures in combination with reverse genetics we will assess antiviral effector mechanisms on the molecular level in the authentic target cells of infection. The specific knock-down of the interferon system in human airway epithelial (HAE) cultures will facilitate virus isolation directly form clinical samples of respiratory viruses that are refractory to isolation in conventional cell lines. **Expected outcomes and perspectives** Our project will contribute to a rapid and robust risk assessment of the consortium. At the interface between molecular studies on virus-host interaction (**P1-5**) and advanced lung tissue and animal models (**P4, P7-9**) we will provide detailed virus phenotypic analyses in the authentic primary lung epithelium. The system will be used to evaluate candidate host and viral target genes discovered within the consortium and is applicable to the risk assessment of any human and animal respiratory virus that may emerge in the future.

1 Working hypothesis and Research question(s)

The human airway epithelium is the major entry port for respiratory viruses and fulfils an important barrier function. In the context of the consortium we will provide our established culture system of human and animal airway epithelia cells to rapidly assess the risk of zoonotic respiratory viruses. This culture system is based on primary human or animal epithelial cells isolated from lung tissue. Following isolation, the primary lung epithelial cells can be expanded and stored. Upon seeding the cells in a trans-well system, applying a specialized differentiation medium, and exposing the apical side to air (ALI cultures), the cells will differentiate to form a pseudostratified epithelial cell layer that morphologically and functionally resembles the airway epithelium *in vivo*.

HAE cultures have recently gained wide recognition since they allow to rapidly assess replication kinetics and cellular tropism of zoonotic respiratory viruses (2-4). Since each virus may differ concerning pathogenicity we expect to see specific biological signatures within the host transcriptome following infection that may correlate with pathogenicity. Likewise, specific virus families have evolved distinct mechanisms to evade early host cell responses, in particular innate immune responses, and we expect to see distinct, i.e. virus family specific, biological signatures within the host transcriptome following infection. Since we use authentic primary target cells, the observed host responses and viral innate immune evasion strategies within the primary airway epithelial cultures are likely identical to virus-host interactions within the first target cells *in vivo*.

The use of transgenic, i.e. lentivirus vector transduced airway epithelial cultures that express particular shRNAs or genes of interest *in trans*, allows furthermore to refine the airway epithelial cultures system to study particular virus-host interactions on the molecular level and to establish IFN reporter cultures that can be used for more specific and accurate risk assessment.

Our work will focus initially on the analysis of MERS-CoV isolates and mutants (**P1**) and will subsequently extended towards the inclusion of other respiratory viruses and primary airway cultures derived from selected livestock and relevant reservoir animals.

2 Own previous work and publications

During the past years we have been involved in studying coronavirus-host interactions with a particular focus on innate immune responses. We have previously shown that coronaviruses efficiently evade Mda5-mediated RNA sensing by involving the conserved coronaviral 2'O-methyltransferase activity (1). Moreover, the establishment of primary HAE cultures allowed us to study the requirements of host cell-encoded proteases for coronavirus entry (2) and we have been involved in the identification of the MERS-CoV entry receptor DPP4 where we could show that DPP4 is expressed on natural primary epithelial target cells (3) (Fig. 1). We could also provide the first evaluation of MERS-CoV replication in primary HAE cells which revealed that MERS-CoV is already capable to efficiently replicate in the human airway epithelium without any further need for adaptation and that MERS-CoV is still vulnerable to the antiviral activity of type I and III interferon (4). Finally, we identified and evaluated a novel type of coronavirus inhibitor that targets membrane-bound RNA synthesis and shows efficacy against all known coronaviruses including SARS-CoV and MERS-CoV (5).

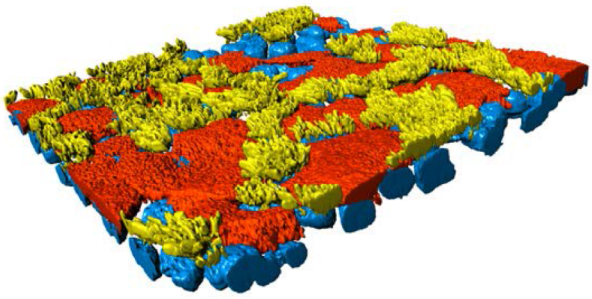


Fig. 1: 3D reconstruction of a primary HAE culture stained for cilia (yellow), nuclei (blue) and the MERS-CoV receptor DPP4 (adapted form Nature cover image , Vol. 495, March 14th, 2013).

2.3. List of project related publications (*=outcome from BMBF SARS consortium)

1. Züst R, Cervantes-Barragan L, Habjan M, Maier R, Neuman BW, Ziebuhr J, Szretter KJ, Baker SC, Barchet W, Diamond MS, Siddell SG, Ludewig B, Thiel V. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. ****Nat Immunol***. 2011;12(2):137-43.
2. Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, Welsch K, Winkler M, Schneider H, Hofmann-Winkler H, Thiel V, Pöhlmann S. TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. ****J Virol.*** 2013 Jun;87(11):6150-60.
3. Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. ****Nature***. 2013 Mar 14;495(7440):251-4.

4. Kindler E, Jónsdóttir HR, Muth D, Hamming OJ, Hartmann R, Rodriguez R, Geffers R, Fouchier RA, Drosten C, Müller MA, Dijkman R, Thiel V. Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential. ****MBio***. 2013 Feb 19;4(1):e00611-12.
5. Lundin A, Dijkman R, Bergström T, Kann N, Adamiak B, Hannoun C, Kindler E, Jónsdóttir HR, Muth D, Kint J, Forlenza M, Müller MA, Drosten C, Thiel V (shared senior author), Trybala E. Targeting membrane-bound viral RNA synthesis reveals potent inhibition of diverse coronaviruses including the middle East respiratory syndrome virus. ****PLoS Pathog***. 2014 May 29;10(5):e1004166.

3 Work plan including milestones

3.1. Aims

The aim of our project is to set up a platform system of airway epithelial cultures derived from animals and humans and further develop this system by genetic modification for rapid assessment of the host cell responses to respiratory virus infection. MERS-CoV isolates and mutants will be used initially, but the system will be applicable to any respiratory virus.

3.2. Work planning

3.2.1. Step 1 – Characterization of host epithelial cell responses to respiratory virus infection

3.2.1.1. Description of work

The initial host response at the entry port of infection is decisive for disease severity, virus spread and virus transmission. We therefore will analyze respiratory virus replication kinetics (initially selected MERS-CoV isolates and mutants) and host epithelial cell responses in HAE cultures. We will do this in close collaboration with **P1**, **P4**, **P5** and **P7**, in order to obtain a comprehensive and comparative view on virus host interaction in various cell culture and lung tissue systems.

We will specifically assess the expression of a panel of cytokines and interferon-stimulated genes (ISGs) that are markers for respiratory virus infection based on our previous transcriptome analyses with MERS-CoV, SARS-CoV, HCoV-229E and influenza A viruses. Later during the project we will expand these analyses to include primary airway cultures derived from selected livestock animals (e.g. swine, cattle, camel) and to include prototype viruses of Paramyxo- (e.g. bovine and human RSV), Orthomyxo- (avian, porcine, human influenza A viruses) families as well as other coronaviruses (HCoV-OC43/bovineCoV). We have currently established primary airway epithelial culture systems available from swine, bats, goats, mouse, camelids (alpaca, llama), and we have obtained epithelial cells from further animal species, such as dogs, cats, gorilla, dromedary camels, cattle, chicken, for which we aim to establish primary airway epithelial cultures. This collection of epithelial cells derived fom various animal species can be employed in the project for comparative replication studies if any zoonotic virus may originate from those species.

3.2.1.2. Expected results

We expect to identify a set of marker genes and host cell responses in the airway epithelium that is characteristic for particular respiratory viruses and correlate with virus pathogenicity. We will furthermore establish virus family-specific transcriptome signatures.

3.2.1.3. Milestones and contingency strategy

M1: Characterization of MERS-CoV replication kinetics and host responses to MERS-CoV infection (including specific isolates and mutants) in HAE cultures: month 18

M2: Characterization of replication kinetics and host responses of prototype respiratory viruses in HAE and animal-derived airway cultures: month 24

It is not likely that this project part will fail. IFN and cytokine profiles will be compared to those obtained in **P5** and assessed if they correlate with the expected virulence of specific viruses (**P1**). One minor risk exists concerning airway cultures from those species that we have not yet established. However, since the main focus is initially on human airway cultures they are not descisive for the overall outcome of the project.

3.2.2. Step 2 – Genetic modification of airway cultures

3.2.2.1. Description of work

In order to assess virus replication and host IFN response kinetics in more detail we will establish HAE IFN reporter cultures. We have recently been successful in establishing genetically modified HAE cultures by employing lentivirus-based vectors for shRNA-mediated knock-down or transgene expression followed by a selection step to obtain homogeniously transduced cultures (H. Jonsdottir, PhD thesis and unpublished data).

In a first step we will employ lentivirus contructs that contain a fluorescence (GFP or mCherry) or luminescence (luciferase) marker gene driven by the IFN-β or Mx (ISG) promoters. This strategy will lead to HAE cultures that allow for fast and simultanious assessment of virus kinetics and spread, and IFN-β (GFP) and ISG (Mx; mCherry) kinetics in a single culture. Likewise, by using two different luciferase reporter genes we can quantitatively assess the IFN-β and ISG expression kinetics. Initially, we will apply these reporter systems to the analysis of interactions of MERS-CoV isolates and mutants within the human airway epithelium. Later during the project, we will also assess the interaction of Paramyxoviruses (RSV) and influenza A viruses.

In a second step we will use lentivirus vectors to specifically knock-down the type-I IFN receptor (IFNAR) in order to assess the impact of host IFN-I responses on MERS-CoV replication in the primary airway epithelium. Importantly, IFNAR knock-down cultures are expected to be superior for virus isolation, which is of particular importance for those viruses that are refractory to isolation in standard cell cultures.

3.2.2.2. Expected results

The establishment of transgenic reporter HAE cultures monitoring IFN expression (IFN-β) and IFN signaling (Mx) will greatly reduce the time to assess the global host IFN response to respiratory virus infection and is applicable to any zoonotic respiratory virus. Likewise, IFNAR knock-down HAE cultures will allow for fast assessment of the impact of host IFN responses on virus replication and will furthermore facilitate virus isolation form clinical samples.

3.2.2.3. Milestones and contingency strategy

M3: Establishment of HAE IFN reporter cultures: month 24
M4: Establishment of IFNAR knock-down HAE cultures: month 36

We do not anticipate any problems concerning the establishment of HAE IFN reporter cultures. Knock-down of IFNAR may be not efficient in the first place, and may require extensive assessment of shRNA efficacy.

3.2.3. Step 3 – Molecular analysis of virus-host interaction in the airway epithelium

3.2.3.1. Description of work

We will initially focus on MERS-CoV isolates and mutants in the context of transgenic HAE cultures. The selection of relevant genes of interest for knock-down or transgene expression will be done in close collaboration with **P4** and **P5**. Later during the project, we will also include selected candidate host genes identified by systems biology approaches performed in **P2** and **P3**. Where applicable and feasible, we will also assess the corresponding molecular interaction in reservoir animal airway cultures (e.g. camelid airway cultures).

3.2.3.2. Expected results

These analyses will validate if specific virus-host interactions detected in immortalized cell lines or by systems biology approaches may apply to the primary natural target cells of respiratory viruses and will further consolidate risk assessment by providing mechanistic insight.

3.2.3.3. Milestones and contingency strategy

M5: Analysis of selected knock-down/transgene expression obtained from **P1/4**: month 36
M6: Analysis of selected knock-down/transgene expression obtained from **P2/3**: month 48

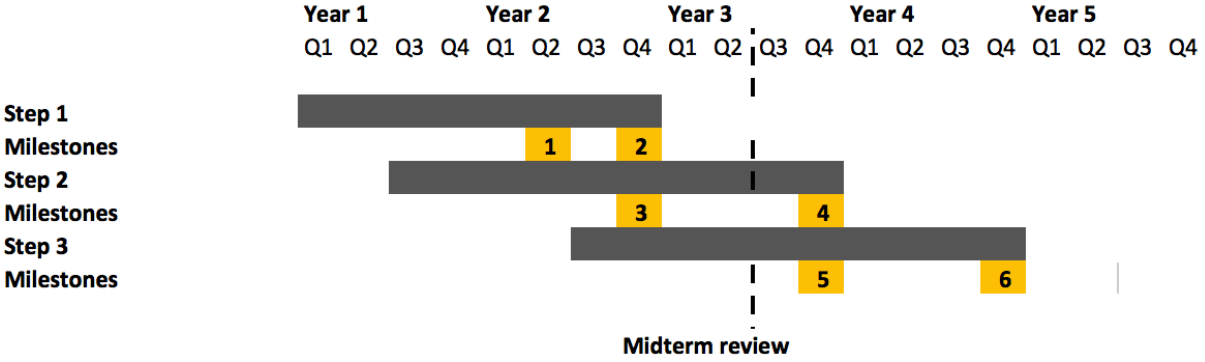
In the highly unlikely case that neither of the projects **P2-5** will deliver genes of interest for the analysis of virus-host interaction in primary transgenic airway cultures we will make a gene-of-interest selection based on our transcriptome analyses.

3.3. Overview of milestones and time planning

3.3.1. Criteria for mid-term evaluation

Until the mid-term evaluation MERS-CoV and prototype respiratory virus replication kinetics and host responses in primary airway epithelial cultures (milestones 1, 2) should be achieved. Furthermore, the IFN reporter airway epithelial culture platform should be established. The work on specific knock-down/transgene expression will be initiated.

3.3.2. Gantt chart



3.4. Considerations regarding dual use of research outcomes

As there are no experiments involved that lead to a gain of function, the project has is no DURC aspect

4 Contribution to the consortium

4.1. Reliance on work in the consortium

The primary airway epithelial culture system will be adapted towards a versatile platform for the consortium to rapidly perform risk assessment of any zoonotic respiratory virus. We will contribute with this system to the assessment of virus-host interaction in the respiratory epithelium in the context of a cross-sectional approach of the consortium. For public health, this represents a relevant and unique experimental risk assessment platform that will be available to reference institutions within and beyond the consortium. By extending this system towards reservoir animals we will contribute to the One Health approach of the consortium in order to assess the risk of transmission and to develop efficacious strategies to combat respiratory virus infections in animals and humans.

4.2. Collaborations within the consortium

Partner	Project area	Type of collaboration
Project 1, Muth, Drosten	Infection phenotyping	Provision of viral strains and mutants
Project 2, Karlas	Risk assessment	Antiviral restriction factor assessment
Project 3, von Brunn	Risk assessment	Assessment of interactions
Project 4, Pöhlmann	Risk assessment	Assessment of TMPRSS2 on virus spread
Project 5, Weber, Ziebuhr	Risk assessment	Assessment of ISGs; technology transfer HAE
Project 7, Hoppe, Hippenstiel, Wolff	Risk assessment	Data comparison with human lung tissue model

5 Quality assurance, standardization, data sharing

We will constantly exchange and compare our data across the consortium, and share our tools and methods. All experimental procedures intended for use as part of the Prepandemic Risk Assessment Platform will be documented in form as standard operating procedures (SOP) which can be added to existing quality management systems at RKI and the University of Bonn.

6 Ethical and legal and considerations

The use of human epithelial cells has been approved by the Ethical Committee of Kanton Bern (Ref.Nr. KEK-BE 302/2015; 2.11.2015). Permissions to work with MERS-CoV isolates and recombinant MERS-CoV have been obtained (Bewilligung A131191; 5.2.2014).

7 Key references (see 3.2)

8 Financial summary (in Euros)

Personnel for 5 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student,	€ 249,760	48	Experimental work as described in chapters 3.2.1, 3.2.2. and 3.2.3..
	Note: SNF Ansatz inkl. Sozialabgaben		
Other resources for 5 years			
Type	Total Budget	Specification / Justification	
Consumables	60,000 €	15'000 € per year in total: RT-PCR reagents; 2000 € Other molecular biology reagents; 3000 € HAE culture media and platic ware; 6000 € Cytokines, antibiotics and other special reagents; 4000 €	
Animal costs	-		
Equipment	-		
Travel	7,500 €	Conferences, visits to other groups, consortium meetings	
Other			
Sum: Total Budget:	317,260 €		
Institutional Overhead:	20%		
Sum: Requested Budget (50% BMBF-share for SME)	380,712 €		

8.1. Note regarding overlap with DZIF

The group is not a member of DZIF. V. Thiel collaborates with C. Drosten under a joint grant within DFG Focus program 1596 ("Ecology and Species Barriers in Emerging Viral Diseases"). There is no work on MERS-CoV in the DFG project. The DFG project is entirely dedicated to human coronavirus 229E, which is neither a subject in the work program of the overall consortium, nor in this specific proposal.

Project No.	7
Title	Prepandemic risk assessment based on human lung tissue
Principal Investigator	Prof. Dr. med. Stefan Hippenstiel Prof. Dr. med. Andreas Hocke Med. Klinik m.S. Infektiologie und Pneumologie Charité – Universitätsmedizin Berlin PD Dr. rer. nat. Thorsten Wolff Robert Koch Institut, Berlin
Contribution to the One Health approach	Based on living human lung tissue, we will define lung-associated criteria of virulence for MERS-coronavirus and other emerging respiratory viruses. Standardization of the lung tissue model with defined infection conditions and severity markers will define a totally novel approach in infection modeling that will be applicable in human and veterinary medicine alike.
Abstract	**Intention and benefit for the consortium and for public health:** Decision making in public health depends on robust and objective information on pathogen virulence. Assessing cross-host (zoonotic) infection processes can be misleading if based on animal models that introduce an irrelevant host into the transmission scenario. The use of live human lung organ cultures for standardized human-specific infection modeling may yield relevant correlates of zoonotic potential. Objective comparisons of replication level and virulence between strains and variants of emerging respiratory viruses may provide a new basis for science-based risk assessment by public health. **Technical highlights, novelty, uniqueness:** Using the unique MERS-CoV expertise and resources of the consortium we will iteratively "train, calibrate, improve, and standardize" the lung infection model and objectively compare surrogates of virulence for relevant MERS-CoV strains with defined/expectable phenotypes. Standard Operation Procedures and virulence scoring criteria will be established. **Expected Outcome and perspectives:** We expect to offer an *ex-vivo* human lung tissue model that provides relevant forecasts of viral epidemic potential After calibration with MERS-CoV, the principle model characteristics allow for the extension to other respiratory zoonotic pathogens.

1 Working hypothesis and Research question(s)

Public health regulations including the German pandemic preparedness plan (*"Pandemieplan")* foresee science-based assessments of zoonotic risks posed by variant (usually zoonotic) respiratory viruses. An inherent characteristic of zoonotic infection is the specific involvement of a distinct animal source species and the human host. Species-specific but mostly unknown factors from both, the pathogen and the target host, determine the zoonotic axis. Therefore, public health laboratory studies on zoonotic diseases must include materials and infection models from the relevant zoonotic partner species, and should avoid animal models outside of the zoonotic axis (1). In addition, the use of clonal cell lines for infection modeling involves limitations such as lack of 3D tissue complexity and cellular interplay.

The lack of clinical knowledge and the absence of suitable infection models reproducing human disease have made it difficult to issue an appropriate public health response to disease importation in Germany and elsewhere (2). Recent studies showed that MERS-CoV circulates in five distinct lineages, partially originating from recombination events, in the dromedary camel population of the Arabic peninsula and Africa (3). Some but not all of these virus lineages have caused human infections, but it is still unclear how and to what extent they compare in virulence and epidemic potential in humans. Serological evidence suggests the presence of MERS-CoV

in camel populations in wide geographic regions with no documented human cases, raising the possibility that such strains have lower zoonotic potential (4,5). However, we are currently lacking experimental tools to examine these aspects. There is an urgent need for species-relevant and robust data to support risk assessments and decision-making in the public health sector. The use of a live *ex-vivo* human lung organ culture for infection with viral variants and novel viruses will provide relevant human infection surrogates. Using the unique resources of the consortium, we will develop these surrogates into score parameters for viral virulence and epidemic potential, integrating the complex interplay between viral replication, host adaption, cellular infection as well as tissue activation and damage, which are all relevant for consideration in public health decisions.
The unique collection of MERS-CoV strains and research materials available through this consortium will be used to "train and calibrate" the model. In an iterative process, Standard Operation Procedures (SOP) will be continuously improved, allowing for systematic comparison of reference and outbreak strains, which will enable classification, relative scoring, and pre-pandemic risk assessment. The model will excellently complement cell culture and animal data and expand our knowledge on viral pathogenicity and disease severity in the human host. This approach guarantees a solid basis for the rapid investigation of emerging viral pathogens and contributes to the prognostic relevance of public health laboratory studies.

2 Own previous work and publications

The RKI group has contributed to the development of MERS-CoV diagnostics and coordinated the public health response to the first cases of MERS-CoV in Germany [e.g., (1, 2)]. Previous studies of the PIs demonstrated the utility of cultured human lung tissue for investigations of circulating, zoonotic and emerging respiratory viruses (3-5). For cell type specific evaluation, spectral confocal microscopy for viral tropism, receptor expression and 3D analysis of tissue damage has been established. In particular, studies on influenza A virus (IAV) demonstrated differences in replication level between avian, porcine, and human-adapted strains in live lung tissue (seasonal and highly pathogenic influenza A virus (IAV) H5N1, as well as novel IAV H7N9). All IAV had a type II alveolar epithelial cell tropism in common (3-4). Further, we demonstrated that MERS-CoV EMC replicated in human lungs (5) comparable to level comparable to the highly pathogenic IAV H5N1, but showed a much broader cellular tropism corresponding well to the DPP4 receptor distribution and alveolar damage pattern. In summary, our data reflect and complement results of the few clinical investigations of MERS-CoV infection available. To the best of our knowledge, the breadth of MERS-CoV strains isolated from human or animal sources has not been evaluated in human lung cultures (neither in clinical studies).

Five project-related publications

1. Corman VM, … Wolff T, Drosten C. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012;17.
2. Buchholz U, …Drosten C., .. Wolff T, …, Haas W. Contact investigation of a case of human novel coronavirus infection treated in a German hospital, October-November 2012. Euro Surveill. 2013;18(8)
3. Weinheimer VK,…, Hippenstiel S, Wolff T, Hocke AC. Influenza A viruses target type II pneumocytes in the human lung. J Infect Dis. 2012;206:1685-94
4. Knepper J,…, Hippenstiel S, Hocke AC, Wolff T. The novel human influenza A(H7N9) virus is naturally adapted to efficient growth in human lung tissue. MBio. 2013;4:e00601-13.
5. Hocke AC,…, Hippenstiel S, Wolff T. Emerging human middle East respiratory syndrome coronavirus causes widespread infection and alveolar damage in human lungs. Am J Respir Crit Care Med. 2013;188:882-6

3 Work plan including milestones

3.1. Aims

We will establish an *ex-vivo* human lung tissue model for standardization and relative scoring of potential virulence to assist public health decision-making in cases and outbreaks of emerging respiratory infections. Parameters in virulence scoring will include viral replication rate, viral cellular tropism, as well as inflammatory activation and damage of host tissue.

3.2. Work planning

Step 1 – Assessment of viral replication, cell infection and host adaption

Score parameters useful as criteria to compare the virulence of given MERS-CoV variants will include viral replication level and cellular infection range in the human alveolar compartment. In previous work, we noted high replication levels of MERS-CoV reference strain EMC and avian IAV H5N1 in human lung tissue. Other avian, porcine, and human IAV strains replicated at significantly different and lower levels. MERS-CoV antigen was visualized in a plethora of cell types of the alveolar compartment, with huge differences in cellular tropism in comparison to IAV infection. We will determine MERS-CoV replication levels in correlation against the number of infected cell types over time (up to 72h). For relative comparison, viral strains will be grouped into (i) strains previously detected in humans (refer to **P1)**: EMC reference strain, 2014 Jeddah outbreak strain, 2015 Korea outbreak strain; (ii) strains isolated from camels or reconstructed to match camel-derived viruses; and (iii) experimentally modified viruses including rEMC-S-D510G and viruses with genomic deletions such as the protein 4a deletion observed in the 2015 Jordan outbreak. Depending on preliminary results in **P1** and **P6**, we will compare original MERS-CoV nsp14^{minus} and passage-adapted MERS-CoV nsp14^{minus} (see **P1**) in human lungs to understand whether MERS-CoV has the potential to adapt to replication in humans. Respiratory viruses other than coronaviruses (source: **P5**) will be tested to validate the transferability of virulence scores to other virus groups. For each virus, the replication level (RT-PCR) as well as the number and types of infected cells will serve to rank strains according to virulence score.
We will start the investigation with the natural variants [Milestone (MS) 1, project month (PM) 18], and expand analysis thereafter to strains circulating in dromedary camels but not humans; analysis of recombinant strains and mediator liberation analysis will be done until PM 39 (MS 2).

Step 2 – Assessment of host tissue activation and damage
The virulence potential of a zoonotic pathogen is reflected by the level of inflammatory tissue activation and damage, which are important for ranking and scoring disease severity. In particular, expression of different anti-viral interferon (IFN) types, their modulatory influence on cyto- and chemokines as well as the extent of alveolar cell damage will be analyzed. By using selected strains as described in step 1 we will assess host factors such as type I IFNα, β, type II IFNγ, and type III IFNλ. These will be correlated with selected pro- and anti-inflammatory cyto-/chemokines (e.g. IL-1β, TNFα, GM-CSF, IL-6, IL-10, COX2/PGE_2 etc.). The selection of target cytokines will be adjusted in accordance to results from project **P5** (innate immune phenotype of viruses). Surrogate markers of alveolar lung barrier damage (e.g. ZO-1, occludin, VE-cadherin) and cellular integrity (caspase-3, LDH, calcein/PI) will be assessed by western blotting, spectral confocal life-tissue imaging and quantitative assays. Additional markers related to proteolytic activation of viruses in tissue will be added according to outcomes of project **P4**. Documentation will include selected 3D reconstruction of affected tissue and further indicators for apoptosis, pyroptosis, or necrosis. For specific questions, electron microscopy will be applied in a complementary manner.
Results will identify (i) strain specific inflammatory host cell activation and (ii) establish markers of critical tissue damage to serve as virulence scoring parameters. At first, we will document tissue activation of circulating strains (PM 21, MS 3), followed by assessment of dromedary camel and recombinant strains. In the same sequence, tissue damage markers will be evaluated (PM 36, MS 4) and analysis will be expanded to specific mutant virus strains (source: **P1**). Respiratory viruses other than coronaviruses (source: **P5**) will be tested in the third phase to validate the transferability of virulence scores to other virus groups.

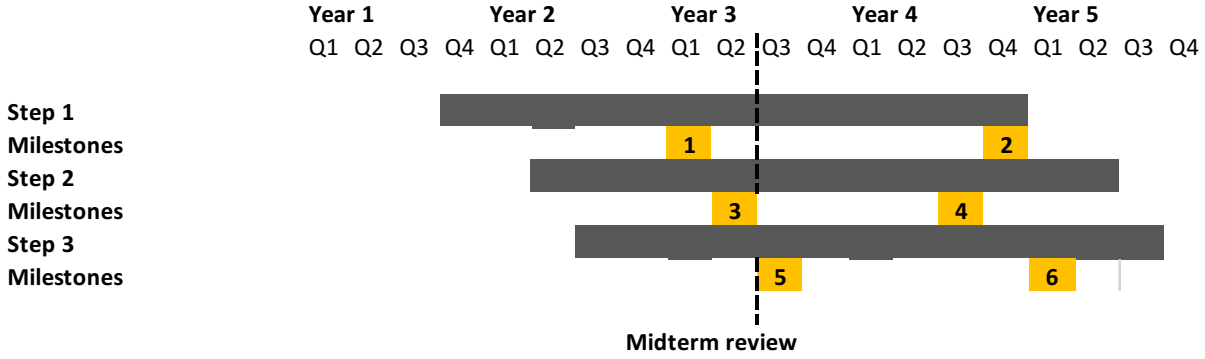
Step 3 – Standard Operations Procedures (SOP) development, and model generalization for assessment of zoonotic virus virulence
In previous studies, we provided first proof of the utility of the human lung model to assess correlates of virulence. However, for risk assessment and decision-making, public health laboratories will require standardized data of controlled quality. Here we will establish and introduce criteria for quality control. We will analyze tissue quality in each case by an established life/dead staining of vital tissue. Parameters to be standardized by exact protocols will include culture conditions, tissue preparation procedures, viral growth, infection techniques and load,

time points, virus readout, materials, etc. We will compare results obtained from specific anti-MERS-CoV monoclonal and polyclonal antibodies with those of a panel of time-stamped patient sera (source: **P1**) to qualify the model for application without specifically-raised antisera that will not be available in case of emergence of a novel virus. The procedures and materials will be documented in SOP, which will be continuously and iteratively improved during the entire project duration. Based on the collected data a combined score will be developed to rank viruses and viral variants according to their projected virulence (refer to steps 1 and 2). Lastly, we will set up SOP for the transfer of some of our criteria to the direct application on patient-derived materials (e.g. broncho-alveolar lavage cells, biopsies, post-mortem samples). Original clinical samples for this purpose are available in the research materials collection at **P1**.
An iterative process will improve SOP with special emphasis, on basic methods for tissue quality control, viral replication and cell infection as well as tissue activation and damage (PM 24, MS 5), and continued to improve and fix all protocols used (incl. patient-derived material). Usage of the results obtained in step 2/3, and of the consortium as well as published results will allow for the establishment of a risk score (PM 42, MS 6).

Step 4 – Implementation at the National Influenza Centre at RKI
In collaboration with **P1, P4, P5,** and **P6** we will implement risk assessment tools including the here-described methodology at the National Influenza Centre (RKI) and at the Consultation Laboratory for Coronviruses (Bonn – moving to Berlin from January 2016). Because the laboratory work in this project will take place in the facilities of RKI, the methodology presented here will be established early.

3.3. Overview of milestones and time planning

Proposed time planning and milestones will allow for the documentation of feasibility within the mid-term evaluation period. More specific experiments (e.g. use of recombinant viruses) will take place during the second period to first secure the presentation of a ready-to-use system at the National influenza centre as soon as possible. Partner RKI will conduct work involving infectious viruses requiring BSL3 conditions (M1-M3), whereas partner Charité is responsible for M4-M6.



3.4. Considerations regarding dual use of research outcomes

This project involves work on a MERS-CoV strain adapted to human cells by serial passaging. The strain (MERS-CoV nsp14^{minus}) will be strongly attenuated and is not expected to gain transmissibility or virulence (for details please see **P1**).

4 Contribution to, and reliance on the consortium

This project provides a unique resource to the common goal of establishing risk assessment capacities for public health laboratories. Implementation depends on unique research materials provided by **P1** and reagents/information on broad markers of viral virulence provided by **P2-P5**. Validation of data with complementary models (**P4, P6**) and future investigation of new important host cell factors (**P2/3**) in human lung will add otherwise unattainable value. Although not part of the here-proposed work program, the methods developed by our project for human lung infection modeling can be used to study lung infection in relevant target animals in veterinary medicine. In particular, we will provide detailed knowledge and resources to the

pathological study within the OneHealth vaccine trial (**P7)**. All our reagents and protocols will be available for adaptation and comparison in camel lung sections.

5 Quality assurance, standardization, data sharing

We will establish SOPs for assessment of zoonotic virus replication, cellular infection, host cell adaption as well as appraisal of activation and damage of human lung tissue allowing for rapid report of high quality human relevant data to the public health system.

6 Ethical and legal and considerations

The local ethics committee approved the use of human lung tissue for this project (incl. data safety) (Reference number: EA2/079/13). The RKI runs a *state-of-the-art* BSL3 laboratory and permission to work with MERS-CoV is granted. We will obtain permission to work with recombinant MERS CoV from local authorities according to the act on genetic engineering.

7 Key references

1. Bean AG et al. Studying immunity to zoonotic diseases in the natural host - keeping it real. Nat Rev Immunol. 2013;13:851-61.
2. Baseler L et al. A Comparative Review of Animal Models of Middle East Respiratory Syndrome Coronavirus Infection. Vet Pathol. 2016;53:521-31.
3. Sabir JS et al. Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. Science. 2016;351:81-4.
4. Chu DK et al. MERS coronaviruses in dromedary camels, Egypt. Emerg Infect Dis. 2014;20:1049-53.
5. Hemida MG et al. MERS coronavirus in dromedary camel herd, Saudi Arabia. Emerg Infect Dis. 2014;20:1231-4.

8 Financial summary (in Euros)

Personnel for 5 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student, 65%, TVÖD	*169,600 €*	*48*	*RKI, Experimental work as described in chapters 3.2, with assistance by 1 technical assistant (staff position).*
PhD student, E13, 65%	*182,480 €*	*48*	*Charité, Experimental work as described in chapters 3.2, with assistance by 1 technical assistant (staff position).*
Other resources for 5 years			
Type	Total Budget	Specification / Justification	
Consumables	*120,000 €*	*Lung culture & processing, sterile plasticware for tissue culture, virus propagation & infection; BSL3-specific protective clothing; primary & secondary antibodies for viral antigen, human lung molecules, live/dead stain, Western blotting, special reagents for microscopy (confocal, EM), antigen retrival, tissue embedding, ELISA (Cyto-/chemokines)*	
Animal costs	-		
Equipment	-		
Travel	*7,500 €*	*Yearly, Annual Meeting Virological Society, Network meetings, German Research Platform for Zoonoses*	
Other	*9,000 €*	*2 Shipments of infectious viruses from Bonn, human lung transport (transplant conditions)*	
Sum: Total Budget:	488,080 €		
Institutional Overhead:	20% for Charité only		
Sum: Requested Budget (50% BMBF-share for SME)	538,976 €		

8.1. Note regarding overlap with DZIF

This institution/group is not a member of DZIF.

Project No. 8	8
Title	Evaluation of MVA-MERS-S immunogenicity and protective efficacy in dromedary camels
Principal Investigator	Dr. med. vet. Asisa Volz Prof. Dr. med. vet. Gerd Sutter Institute of Infectious Diseases and Zoonoses, LMU Munich
Contribution to the One Health approach	Enabling studies to evaluate immunogenicity and protective efficacy of MVA-MERS-S vaccine in dromedary camels for application as veterinary vaccine to inhibit transmission to humans.
Abstract	**Intention and benefit for the consortium** MERS-CoV poses a major challenge as zoonotic pathogen because it naturally circulates in dromedary camels, an important livestock species and sustained source for human infection. Vaccination of the animal reservoir may be most promising to prevent human infections and further spread of MERS-CoV as a pathogen of growing global concern. **Technical highlights, novelty, uniqueness** In a recent proof-of-principle experimental study in dromedary camels we demonstrated immunogenicity and efficacy of MVA-MERS-S, a Modified Vaccinia virus Ankara (MVA) vaccine expressing the MERS-CoV spike protein (Haagmans et al. 2016 Science 351:77-81). Here, we will firstly evaluate immunogenicity and efficacy of MVA-MERS-S immunizations in camels under field conditions. Major objectives are (i) to define the most appropriate application route for the MVA-MERS-S vaccine, (ii) to characterize levels and longevity of immune responses (including T cells) elicited by MVA-MERS-S vaccination, and (iii) to demonstrate the capacity of vaccine induced immunity to prevent the shedding of MERS-CoV. **Expected outcomes and perspectives** These field studies will be essential to better understand MERS-CoV infections in camels, to elucidate on the quality of protective immunity and to identify the relevant immune correlates of protection. This data may help to encourage camel vaccinations as successful approach to reduce the MERS CoV zoonotic burden.

1 Working hypothesis and Research question(s)

Primary MERS-CoV infection in humans is acquired through contact with infected dromedaries. Efficient approaches to prevent primary human infection needs to focus on the camel-human interface providing an example for a typical One Health research setting. Similar to the situation in other zoonotic diseases such as avian influenza or rabies, vaccination of the reservoir animal species may well the method of choice to limit human acquisition. The evaluation of MVA-MERS-S, a candidate vaccine being developed for use in humans, is essential to implement a routine vaccination program in the camel population. The major working hypothesis of this project is that vaccination strategies efficiently prevent the shedding and may even permit eradication of MERS-CoV from the camel population. In doing so, virus transmission to humans will be disrupted.

We will gain further insights into the epidemiology and pathogenicity of MERS-CoV infection in camels by analyzing MERS-CoV specific immunity in a MERS-CoV-endemic camel population. We expect that MVA-MERS-S vaccination protects against MERS-CoV infection in young camels and therefore reduces the MERS-CoV burden within the camel population. Important research questions to be addressed in this immune monitoring project include the identification of the optimal time window for immunization, the definition of feasible methods for application,

and the determination of the numbers of inoculations needed for successful vaccination under field conditions.

2 Own previous work and publications

MVA, a replication deficient and safety tested VACV, serves as a multipurpose viral vector for vaccine development against various infectious diseases and cancers. The applicant has established a technology platform to produce and characterize GMP grade recombinant MVA vaccines in preclinical experiments (Volz et al. 2014, 2015, 2016). Background is that multiple MVA vectors are already being evaluated in clinical trials as vaccines against important infectious diseases including AIDS, malaria, tuberculosis and influenza. Major benefits of MVA include (i) an established record in clinical safety, (ii) longstanding experience in the genetic engineering of the virus, (iii) a large data set demonstrating efficacy in preclinical models with capacity to induce protective antigen-specific antibody and cellular immune responses, and (iv) the availability of virus production under GMP at an industrial scale. Using our MVA technology platform first experimental MVA vector vaccines against the Middle East Respiratory Syndrome (MERS) have been successfully developed within the DZIF (Song et al. 2013; European patent application EP 15 15 1594.7)). Advanced preclinical evaluation extensively characterized the MVA-MERS-S candidate vaccine concerning immunogenicity, efficacy and safety in mice (Volz et al 2015) and in dromedary camels (Haagmans et al. 2016). At the moment, with DZIF funding the applicant is preparing for a phase I/II clinical testing of MVA-MERS-S and first-in-man studies are expected to start in Q4/2016. This previous work provides a useful basis to further develop MVA-MERS-S also as candidate vaccine in camels. The concerted actions of clinical trial evaluation and field studies in camels are ideally suited to accomplish the One Health approach.

Five project-related publications

Haagmans BL, van den Brand JM, Raj VS, Volz A, et al., Baumgärtner W, Segalés J, Sutter G, Osterhaus AD. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science. 2016 Jan 1;351(6268):77-81.

Volz A, Lim S, Kaserer M, Lülf A, Marr L, Jany S, Deeg CA, Pijlman GP, Koraka P, Osterhaus AD, Martina BE, Sutter G. Immunogenicity and protective efficacy of recombinant Modified Vaccinia virus Ankara candidate vaccines delivering West Nile virus envelope antigens. Vaccine. 2016 Apr 7;34(16):1915-26

Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, Schmidt J, Becker C, Eickmann M, Becker S, Sutter G. Protective Efficacy of Recombinant Modified Vaccinia Virus Ankara Delivering Middle East Respiratory Syndrome Coronavirus Spike Glycoprotein. J Virol. 2015 Aug;89(16):8651-6.

Volz A, Langenmayer M, Jany S, Kalinke U, Sutter G. Rapid expansion of CD8+ T cells in wild-type and type I interferon receptor-deficient mice correlates with protection after low-dose emergency immunization with modified vaccinia virus Ankara. J Virol. 2014 Sep;88(18):10946-57.

Song F, Fux R, Provacia LB, Volz A, Eickmann M, Becker S, Osterhaus AD, Haagmans BL, Sutter G. Middle East respiratory syndrome coronavirus spike protein delivered by modified vaccinia virus Ankara efficiently induces virus-neutralizing antibodies. J Virol. 2013 Nov;87(21):11950-4.

3. Work plan including milestones

3.1. Aims

The overall aim of this work package is to induce high level protective immunity against MERS-CoV infection in dromedary camels. We will newly establish and run the immune assays for best possible characterization of the immune responses induced following MVA-MERS-S vaccinations. These immunological data will help us to select the optimal vaccination strategy and to establish a practicable and efficient routine vaccination program for dromedary camels in endemic areas.

3.2. Work planning

Step 1

***Establishment* of immune assays for virus specific immunity in camels**

Preliminary data suggest that the induction of MERS-CoV neutralizing antibodies is associated with significant reductions of MERS-CoV loads in nasal excretions after high-dose experimental infection (Haagmans et al. 2016). However, in this context we still lack understanding about the time period in which young camels mount protective levels of MERS-CoV specific antibodies after vaccination, and how long these protective antibodies are maintained. Thus, we will further characterize kinetics and quality of MERS-CoV specific antibodies, investigate the corresponding antibody subpopulations, and elucidate the involvement of cellular immunity. To

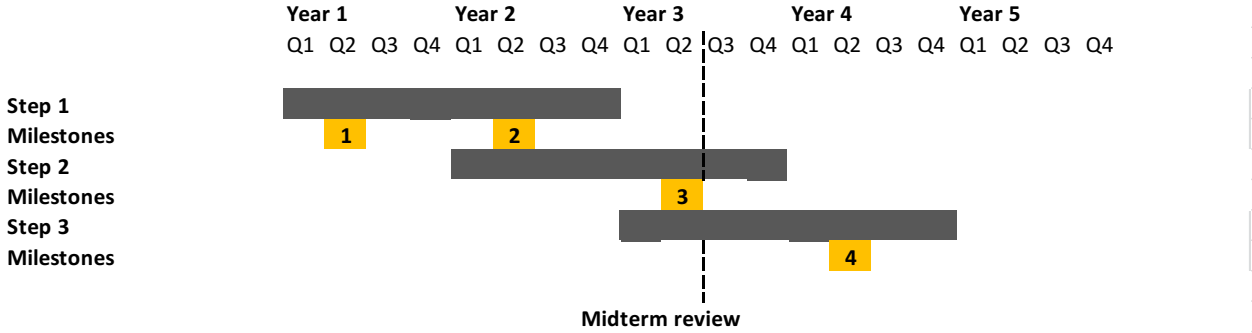
initially test for MVA-MERS-S immunogenicity we will determine MERS-CoV binding antibodies using well-established ELISA (Meyer et al. 2016) and serum neutralization assays (Meyer et al. 2016 and Haagmans et al. 2016). With regard to possible differences in the immune recognition of MERS-CoV variants, we will analyze the capacity of vaccine-induced antibodies to neutralize MERS-CoVs isolated from different outbreaks (strains provided by **P1**).
In addition to conventional IgG antibodies (four heavy chains and antigen-binding site consisting of two heavy-/light-chain type variable domains), camels also produce simpler antibodies consisting only of two single heavy-chains (so called heavy-chain only immunoglobulin G-like antibodies). Here, the antigen-binding site is made by a single heavy-chain variable fragment. These camelid heavy-chain antibodies are known to have a particularly high affinity and specificity for their specific target antigen, and appear to be capable of binding epitopes that are hidden within protein structures. Yet, about 50% of the antibodies in dromedary camels are of the ordinary mammalian heavy/light-chain type, and with regard to MERS-CoV neutralization we lack the knowledge about the relative contribution of these different antibody types.
In addition, we know little about cellular immunity in camelids and the impact of cellular responses to infections in general. Overall, T cell immunity is increasingly known to play a critical role in vaccine-mediated rapid protection against viral diseases as suggested by evidence from preclinical disease models in other species. This seems to be even more important in light of the fact that antibody responses alone might not be fast and sustained enough to control MERS-CoV infection (important to design vaccine-based outbreak interventions in humans and camels alike). It will be important to characterize the cellular MERS-CoV-specific immune responses in dromedary camels after MVA-MERS-S vaccination. However, methodology to determine cellular immunity in camels is not well established and needs to be adapted from related species for the purpose of this project. Isolation and cryopreservation of camel peripheral blood mononuclear cells (PBMC) will be achieved by established protocols for Ficoll density gradient centrifugation, freezing and storage in liquid nitrogen. To screen for the presence of MERS-CoV-specific T cells we will analyze PBMC in established standard proliferation assays (e.g. 3H-thymidine incorporation) in response to *in-vitro* stimulation with MERS-CoV antigens. In addition, the cDNA sequences of the dromedary camel cytokines IFN-γ, IL-2 and IL-4 are available (Nagarajan et al. 2012). Thus, it will be possible to develop cytokine-based diagnostics by RT-PCR to more specifically analyze antigen-specific T cell responses using PBMC cultures. Cytokine mRNA quantification by droplet digital RT-PCR will be established to allow for highly precise comparison of cytokine expression in response to antigen stimulation in these cultures (Hindson et al. 2013). Moreover, we aim to establish specific assays for the quantification of cytokine-producing T cells in the PBMC from camels. This will require the development or use of appropriate antibody reagents to camel IFN-γ or IL-2. Based on these antibodies we plan to adapt our well-established ELISPOT methodology to analyze antigen-specific T cells from camels. These assays will then be further validated for the evaluation of MERS-CoV-specific T cell responses.
Expected results: Innovative methodological repertoire of assays that allow for detailed characterization of immune responses in dromedary camels.
Milestone 1 (month 6): Established ELISA and neutralization assays to assess MERS-CoV-specific antibody responses
Milestone 2 (month 18): Established lymphocyte proliferation assays and/or analysis of cytokine expression (IFN-γ / IL-2).

Step 2 – Immunogenicity of MVA-MERS-S in dromedary camels
Preliminary experimental data support immunogenicity and efficacy of MVA-MERS-S in dromedary camels (Haagmans et al. 2016). Based on the work of **P10**, the practicability of camel vaccination with MVA-MERS-S will be tested in our consortium in field studies involving naturally occurring MERS-CoV infections in newborn camel calves. The study will comparatively assess application routes for MVA-MERS-S immunization in the field. **Study design in brief (refer to P10 description):** Immunization experiments in four different experimental groups (n=12 pairs/group) involves two vaccine applications (3x10E8 pfu/route MVA-MERS-S) in eight-week intervals (week 0 and week 8, starting at month 4 after parturition).
Sampling: bleedings day 0, then 14 day intervals; 3 nasal swabs/week (each with wet and dry swabbing)

Analysis: MERS-specific antibodies will be comparatively analyzed from serum samples by ELISA and VNT assays as established in step 1. We will further characterize the MERS-CoV specific antibodies activated by MVA-MERS-S vaccination including the testing with different MERS-CoV variants in collaboration with project **P1**. We will further test for the quantity and the functional activity of heavy-chain only IgGs (hcAbs) in neutralizing different MERS-CoV variants in collaboration with **P1**. Using these vaccine-induced hcAbs may provide innovative preventive and/or therapeutic approaches to MERS.
To further evaluate the immunogenicity of the different application routes, we will test the activation of MERS CoV specific T cell responses. For this peripheral blood mononuclear cells (PBMC) isolated from blood samples will be analyzed by using lymphocyte proliferation assays, droplet digital PCR for cytokine expression IFN-γ / IL-2 and ELISPOT assays as established in step 1. Additionally, important efficacy data will be collected by comparative testing for MERS-CoV shedding in the different study groups. For this, virus loads (infectious MERS-CoV and MERS-CoV RNA) will be analyzed as described previously (Haagmanns et al. 2016). In addition, analysis of orthopoxvirus-specific immune responses (MVA/Camelpox virus-specific antibodies) will allow to prove successful immunization and may further support use of MVA-MERS-S as bivalent MERS/Camelpox vaccine in dromedary camels.
Expected results: Activation of MERS specific immunity in camels, reduction of MERS-CoV burden within the cohort, detection of differences in qualities and levels of MERS-CoV specific immune responses when comparing the three different modes of MVA-MERS-S application.
Milestone 3 (30 months): Seroconversion in camels, monitoring of MVA-MERS-S immunogenicity.

Step 3 – MVA-MERS-S dose reduction immunization study in dromedary camels This study will generate important results with regard to practicability of camel vaccination. Design will depend on the outcome of the experiments in step 2. A single application of MVA-MERS-S could be sufficient for a substantial reduction of MERS-CoV excretion from juvenile dromedary camels in the first year of life, with induction of robust MERS-CoV specific immunity. This might be of enormous importance for the development of future emergency vaccination strategies. Single-shot vaccination would greatly facilitate the practical management of vaccinations in larger camel populations in the field. Together with **P10**, we will therefore evaluate the immunogenicity and efficacy after single vaccination using the most efficient application route as determined in step 2.
Study design: refer to **P10** description.
Sampling: bleedings day 0, then 14 day intervals; 3 nasal swabs/week (each with wet and dry swabbing). **Analysis**: as described in step 2, evaluation will focus on the efficacy of the single immunization compared to the prime-boost immunization schedule as already characterized in step 2.
Expected results and Milestone 4 (48 months): Definition of optimized immunization strategy in dromedary camels.

3.3. Overview of milestones and time planning



3.4.DURC safety assessment

The experimental work in this project has no relevance with regard to DURC.

4 Contribution to the consortium

This project essentially relies on collaboration partners in Projects **P1**, **P9** and **P10**. We will very closely interact with our colleagues in Dubai Dres. Wernery/Nagy to enable the field studies in juvenile dromedary camels. Nina Söllner, the DVM/PhD student working on the pathological workup in Dubai during years 2 and 3 in **P9**, is well acquainted with our group.

5 Quality assurance, standardization, data sharing

In our project, we will establish dedicated SOPs for the immune monitoring of vaccine induced immune responses in dromedary camels.

6 Ethical and legal and considerations

All animal experiments are reviewed and approved by the Animal Ethic Committee of the Central Veterinary Research Laboratory (CVRL) and the Ministry of Climate Change and Environment (MOCCAE) of the United Arab Emirates (Permit Number: 550353).

7 Key references

1. Meyer B, et al., Wernery R, Drosten C. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014 Apr;20(4):552-9.
2. Wernery U, et al., Drosten C. Acute Middle East respiratory syndrome coronavirus infection in livestock Dromedaries, Dubai, 2014. Emerg Infect Dis. 2015 Jun;21(6):1019-22
3. Widagdo W, et al., Baumgärtner W, Osterhaus AD, Koopmans MP, van den Brand JM, Haagmans BL. Differential expression of the Middle East respiratory syndrome coronavirus receptor in the upper respiratory tracts of humans and dromedary camels. J Virol. 2016 Apr 14;90(9):4838-42.
4. Corman VM et al., Drosten C, Müller MA.. Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992-2013. Emerg Infect Dis. 2014 Aug;20(8):1319-22
5. Nagarajan, G. et al. (2012). Cloning and sequence analysis of IL-2, IL-4 and IFN-γ from Indian Dromedary camels (Camelus dromedarius). Research in Veterinary Science 92, 420.
Hindson, C. M. , Chevillet, J. R. , Briggs, H. A. , Gallichotte, E. N. , Ruf, I. K. , Hindson, B. J. , Vessella, R. L., and Tewari, M. (2013). Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Meth 10, 1003.

8 Financial summary (in Euros)

Personnel for 4 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
PhD student / E13 50%	*138,000*	*48*	

Other resources for 4 years		
Type	Total Budget	Specification / Justification
Consumables	*83,000*	*Camel-specific immune assay development: ELISA, VNT, analysis of IgG-like hcAbs, cell proliferation assays, ddPCR analysis of cytokine expression, cytokine cloning and expression, ELISPOT development;*
Animal costs	*--*	*Will be covered by CVRL and MOCCAE*
Equipment	*--*	*--*
Travel	*4,000*	*Consortium meetings; GfV Annual Meeting for data presentation.*
Other	*3,000*	*Shipments Munich-Dubai-Munich (partial cost only)*

Sum: Total Budget:	229,000
Institutional Overhead:	20%
Sum: Requested Budget (50% BMBF-share for SME)	274,800

8.1. Notes regarding overlap with DZIF

The MVA-MERS-S vaccine has been developed by us in DZIF, and will be studied for human application only. All DZIF project outcomes feed synergistically into the present project. There are no overlaps as DZIF does not focus on veterinary application.

Project No.	9
Title	Pathological validation of the success of MVA-MERS-S vaccination in dromedary camels
Principal Investigator	Dr. med. vet. Vanessa Herder, PhD Prof. Dr. med. vet. Wolfgang Baumgärtner, PhD. Department of Pathology Prof. Dr. med. vet. Albert D. M. E. Osterhaus Research Center for Emerging Infections and Zoonoses University of Veterinary Medicine Hannover Bünteweg 17 30559 Hannover, Germany
Contribution to the One Health approach	Vaccination should aim at reduction of tissue damage and virus replication, thereby preventing disease in animals and humans. The success of MERS-CoV vaccination will be proven under field conditions by evaluation of tissue pathology of vaccinated vs. non-vaccinated animals after natural MERS-CoV exposure. Reduction of human zoonotic exposure through MERS-CoV eradication from the camel reservoir will provide a major proof of the One Health concept.
Abstract	The prevention of Middle East respiratory syndrome (MERS)-coronavirus (CoV) infection in camels by vaccination has been proven under experimental conditions, involving a dual application mode (combined intramuscular and mucosal application). The most relevant next steps will involve optimizations and simplifications of the immunization scheme, as well as proof of immunity under conditions of natural exposure. Detailed pathological workup and comparison of vaccinated vs. non-vaccinated camels will form an elemental part of these studies. Whether vaccination will lead to limited virus dissemination and reduced inflammatory reactions in immunized animals will therefore be investigated by pathological workup in this project. All obtained pathological findings will be correlated with data on virus shedding in saliva and nasal swabs determined during the vaccination trial.

1 Working hypothesis and Research question(s)

Middle East respiratory syndrome (MERS) is a severe zoonotic infection of the human respiratory tract that is acquired through contact with camels [1]. Vaccinating animals may the the most effective means to limit intra- and cross-species transmission [2].

Experimentally infected camels shed large quantities of infectious virus in the upper respiratory tract. Inflammation and virus antigen can be detected in the upper respiratory tract in affected animals [3]. Vaccination under experimental conditions has been shown to reduce virus shedding [4]. Whether this vaccination strategy is also successful after natural virus exposure is unknown at present. The present project connects to the vaccination experiments by projects **P8** and **P10**. The pathological investigation complements the results of the vaccination studies and **aims** to validate whether the vaccination is able to:

1) reduce the virus load in the upper respiratory tract,
2) reduce or eliminate the virus in the lymphoid organs, like mesentery lymph nodes,
3) reduce the virus-induced pathology, like inflammation and tissue damage and
4) define which of the applications, dosages and schedules of vaccination show the most effective results regarding reduced virus load, virus distribution and pathomorphological changes in the camels.

Pathological findings in tissues of all treatment groups will be comparatively analyzed according to these criteria, providing **insight** about the safest vaccination strategy.

We hope that at least one vaccination strategy will be able to:

1) cause complete absence of virus replication in the upper respiratory tract,

2) cause a lack of tissue damage,
3) prevent virus spread to lymphoid organs, such as the mesenteric lymph nodes,
4) be efficient beside the absence of any adverse reactions, like local vaccination reactions including inflammation, or systemic side effects like fever or severe immunosuppression

2 Own previous work and publications

Dr. V. Herder, PhD, has a long standing expertise in veterinary pathology with a focus on viral pathogenesis in experimental and natural virus infection in various species, such as mice and ruminants (1-3). Among her key skills are methods to characterize the distribution of viral antigens in tissue, as well as studies of inflammation including the phenotypic characterization of inflammatory cells. She has specific experience identifying degenerative changes in natural and experimental virus infections (1,2). She has a experience in experimental animal studies including the comparison of results from different treatment groups (3). She is co-affiliated with the groups of Proff. Baumgärtner and Osterhaus at TiHo. Both groups have collaborated on the pathogenesis as well as morphological and immunophenotypical characterization of lesions induced by MERS-CoV in camels (4,5).

Nina Söllner, DVM, will complete her DVM thesis under this project. She has graduated in veterinary medicine from LMU Munich in 2016 and is already familiar with the infrastructure and key researchers in Dubai, having stayed there for a preparatory practical. She will be on-site for the full 24 months to take part in necropsies and help implementing the overall work plan.

List of five project-related publications:

1. **Herder V**, Hansmann F, Wohlsein P, Peters M, Varela M, Palmarini M, **Baumgärtner W**. Immunophenotyping of inflammatory cells associated with Schmallenberg virus infection of the central nervous system of ruminants. PLoS One. 2013 May 7;8(5):e62939.
2. Hahn K, Habierski A, **Herder V,** Wohlsein P, Peters M, Hansmann F, **Baumgärtner W.** Schmallenberg virus in central nervous system of ruminants. Emerg Infect Dis. 2013 Jan;19(1):154-5.
3. **Herder V**, Hansmann F, Stangel M, Schaudien D, Rohn K, **Baumgärtner W,** Beineke A. Cuprizone inhibits demyelinating leukomyelitis by reducing immune responses without virus exacerbation in an infectious model of multiple sclerosis. J Neuroimmunol. 2012 Mar;244(1-2):84-93.
4. Widagdo W, Raj VS, Schipper D, Kolijn K, van Leenders GJ, Bosch BJ, Bensaid A, Segalés J, **Baumgärtner W**, **Osterhaus AD**, Koopmans MP, van den Brand JM, Haagmans BL. Differential Expression of the Middle East Respiratory Syndrome Coronavirus Receptor in the Upper Respiratory Tracts of Humans and Dromedary Camels. J Virol. 2016 Apr 14;90(9):4838-42.
5. Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, Schipper D, Bestebroer TM, Okba N, Fux R, Bensaid A, Solanes Foz D, Kuiken T, **Baumgärtner W**, Segalés J, Sutter G, **Osterhaus AD**. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science. 2016 Jan 1;351(6268):77-81.

3 Work plan including milestones

3.1. Aims

The project aims at the pathological characterization of pathogenesis and vaccine-dependent protection of camels against natural infection with MERS-CoV. Special emphasis will be given to viral load and tissue distribution with and without vaccination. Furthermore, induced pathological lesions in the respiratory tract and adverse reactions will be characterized.

3.2. Work plan

All animals used in these experiments will originate from the planned vaccination study outlined in project **P10**. The pathological investigation will focus on experimental step 2 (**P10**). Four months after parturition, all camel calves will be vaccinated for the first time (week 0) followed by a second vaccination 8 weeks later (double vaccination strategy). The candidate vaccine MVA-MERS-S, which is approved for a clinical phase I trial in humans, will be used in this approach. The study consists of 4 different treatment groups (group 1 – 4). Each group will comprise 12 pairs of animals (dam and calf). Group 1 will be double sham vaccinated, and group 2 will be vaccinated two times intramuscularly (i.m.) and intranasally (i.n.); group 3 will be vaccinated two times i.m.; group 4 will be vaccinated i.n. only (**P10**). For the pathological investigation, 3 randomly chosen camel calves out of each group will be necropsied. An overview on the experimental setting is provided in **Table 1**.

Table 1. Study design for necropsies (week 0 and week 8).

Group 1: Sham-inoculated controls	**Group 2: Intramuscular and intranasal vaccination**	**Group 3: Intramuscular vaccination**	**Group 4: Intranasal vaccination**
3 camel calves for necropsy (out of 12 pairs of animals)	3 camel calves for necropsy (out of 12 pairs of animals)	3 camel calves for necropsy (out of 12 pairs of animals)	3 camel calves for necropsy (out of 12 pairs of animals)
12* camel calves for necropsy			

*In case of calves of group 2-4 showing clinical signs of MERS-CoV-infection and which are not selected for necropsy, these animals will be added to a maximum of 1 animal per group leading to maximum 15 animals.

Necropsies will be performed two days after the animals have been tested positive for MERS-CoV applying an immunochromatographic assay for the detection of MERS-CoV antigen in nasal swabs of infected animals [5]. In case of a negative virus antigen test in camel calves of groups 2 to 4, these animals will be necropsied at a fixed point of time, 12 weeks after the second immunization. In case camel calves show clinical signs of a MERS-CoV infection in group 2-4, these animals will be included in the necropsy study. In this case, a maximum of one animal per group will be necropsied, requiring a maximum of 15 necropsies instead of 12.
Tissue samples for downstream investigations will be taken during all necropsies. Downstream investigations will be divided into 3 steps (milestones). Before the start of these investigations step 1 of project **P10** (*"MERS-CoV-specific monitoring for seroconversion/infection in dromedary camels")* has to be completed.

3.3. Step 1 experiment (necropsy & sampling)

V. Herden as well as the PhD student will conduct necropsies jointly with Prof. J. Kinne at CVRL. After anesthesia, animals will be humanely killed and immediately tissue samples of all organ systems and tissues will be taken for formalin fixation (histology), or will be snap frozen (for molecular analyses or immunofluorescence). After fixation, tissue specimens will be paraffin-embedded. Blocks will be used for preparing histological sections of 3-5µm thickness, and mounted on glass slides for hematoxylin and eosin staining. For detection of viral protein and nucleic acids, immunohistochemistry, immunofluorescence or *in situ*-hybridization will be performed, respectively [6].
Milestone 1 (project month 24): Necropsy and sampling of tissues of all organs of the different treatment groups.

3.4. Step 2 experiment (histology, immunohistochemistry, immunofluorescence and *in situ*-hybridization, data evaluation)

Relevant localizations of virus replication in the upper and lower respiratory tract as well as within the lymph nodes will be investigated to evaluate tissue damage and inflammation [3]. In addition, immunohistochemistry, immunofluorescence of virus antigen, and *in situ*-hybridization for viral nucleic acids will be done to provide insights into the presence and replication of the virus in vaccinated and non-vaccinated animals. Where applicable, histochemical immunphenotyping of inflammatory cells will be performed to characterize the immune response.
Milestone 2 (project month 36): Histological and immunohistochemical investigation and evaluation of tissues obtained during necropsy

3.5. Overview of milestones and time planning

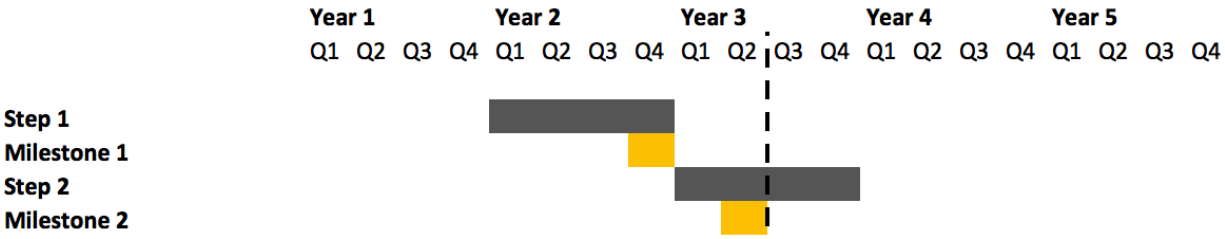
The project is planned for two years, starting at month 13 of the vaccination trial (**P10**). After determination of the optimal time window for immunization by **P10** the vaccination and necropsy time points will be appointed. The exact time schedule of this project depends on the results of **milestone 1 of P10** "*MERS-CoV-specific monitoring for seroconversion/infection in dromedary camels*". Tentatively, after the first year (month 24 of **P10**) the necropsies, sampling, cutting and staining of all tissues will be finished. Within the following year, all histopathological and immunohistochemical investigations will be finished and evaluated. After comparison of the

obtained data with results of clinical and virological investigations, the study will be finished at month 36 of the overall funding period.

3.5.1. Criteria for mid-term evaluation

It is planned to finish the necropsies, sampling, cutting and staining of all tissues and organs from all investigated animals until the mid-term review. Preliminary data from the evaluation of histology, immunohistochemistry, immunofluorescence and *in situ*-hybridization will be available by that time (Gantt chart (3.5.2)).

3.5.2. Gantt chart



3.6. Considerations regarding dual use of research outcomes

The work of these experiments is not related to Dual Use Research of Concern (DURC).

4 Contribution to the consortium

The feasibility of a MVA-MERS-S vaccination in the field will be tested by project **P10** (Nagy/Wernery), and the immune components linked to the reduction of virus shedding will be investigated in additional collaboration with project **P8** (Volz/Sutter). The pathomorphological and histological work will complement the vaccination trial by showing which immunization scheme shows the least if any adverse effects, and which vaccination procedure will be best in reducing or preventing disease.

4.1. Reliance on work in the consortium

This work package depends on the conduction of the vaccination trial in Dubai (**P10**) and the collaborative evaluation of vaccination outcomes with **P8** and **P10**. Tools to detect virus by RT-PCR, monoclonal antibodies against different viral proteins, polyclonal human and camel sera, as well as other regents as required will be obtained from **P1**. P1 also contributes prior knowledge on the natural course of infection gathered in several earlier joint studies with the **P10** principal investigators. Specific collaborations to characterize details of lung pathology in comparison with human infection will be implemented with **P7**, providing preliminary findings as well as histomorphological reagents and techniques developed for their technically advanced human lung infection model.

4.2. Collaborations within the consortium

Partner	Project area	Type of collaboration
Project 10, Field study on the feasibility of MVA-MERS-S vaccination in dromedary camels (Nagy/Wernery)	Vaccination trial	In this project some of the animals of the immunization study will be used for necropsies.
Project 8, Immunemonitoring and virus testing (Volz/Sutter)	Vaccination trial	Data obtained from the necropsy study will be compared to the results of amount of virus shedding in nasal swabs.

5 Quality assurance, standardization, data sharing

Scientists working at the University of Veterinary Medicine Hannover are obliged to uphold the general principles of scientific work, like observation of the rules of the medical arts; documentation of results; consistent questioning and critical review of all of one's own results; strict honesty in regard to the work of co-workers, competitors, and predecessors. Obtained data will be stored for 10 years in two independent places. Originality and quality of work always have precedence over quantity. Based on the obtained data, an inventory of methods and protocols will be prepared and first standardization data will be generated and provided to all partners. Finally, an optimal standard protocol for all steps of sample processing for histology,

immunohistochemistry, immunofluorescence and *is situ*-hybridization will be available and shared with all partners.

6 Ethical and legal and considerations

All animal experiments are reviewed and approved by the Animal Ethic Committee of the Central Veterinary Research Laboratory (CVRL) and the Ministry of Climate Change and Environment (MOCCAE) of the United Arab Emirates (Permit Number: 550353).

7 Key references

1. Yu IT, Li Y, Wong TW, Tam W, Chan AT, et al. (2004) Evidence of airborne transmission of the severe acute respiratory syndrome virus. N Engl J Med 350: 1731-1739.
2. Perlman S, Vijay R (2016) Middle East respiratory syndrome vaccines. Int J Infect Dis.
3. Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, et al. (2014) Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. Emerg Infect Dis 20: 1999-2005.
4. Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, et al. (2016) An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science 351: 77-81.
5. Song D, Ha G, Serhan W, Eltahir Y, Yusof M, et al. (2015) Development and validation of a rapid immunochromatographic assay for detection of Middle East respiratory syndrome coronavirus antigen in dromedary camels. J Clin Microbiol 53: 11781182.
6. Hahn K, Habierski A, Herder V, Wohlsein P, Peters M, et al. (2013) Schmallenberg Virus in Central Nervous System of Ruminants. Emerging Infectious Diseases 19: 154-155.

8 Financial summary (in Euros)

Personnel for 5 years

Position / Salary Group	Total Budget (€)	Duration (months)	Tasks / Justification
PhD student, E13, 65%	79,450	24	Nina Söllner, DVM, will complete her DVM thesis under this project. She has graduated in veterinary medicine from LMU Munich in 2016 and is already familiar with the infrastructure and key researchers in Dubai, having stayed there for a preparatory practical. She will be on-site for the full 24 months to take part in necropsies and help implementing the overall work plan.

Other resources for 5 years

Type	Total Budget	Specification / Justification
Consumables	-	Material for histology (formalin, paraffin, glass slides), immunohistochemistry, immunofluorescence, *in situ*-hybridization (antibodies, staining material): *Will be covered by Central Veterinary Research Laboratory (CVRL) and the Ministry of Climate Change and Environment (MOCCAE) of the United Arab Emirates:*
Animal costs	-	*Will be covered by CVRL and MOCCAE*
Equipment	-	
Travel	*7000*	Yearly visits of Dubai, costs for flight tickets; *costs for accommodation will be covered by CVRL and MOCCAE*
Other	-	*Shipments of formalin-fixed and paraffin-embedded material from Dubai to Hannover: Will be covered by CVRL and MOCCAE*

Sum: Total Budget:	86,450
Institutional Overhead:	20%
Sum: Requested Budget (50% BMBF-share for SME)	103.740

8.1. Note regarding overlap with DZIF

This group is not a member of DZIF.

Project No.	10 – Overseas project with external funding
Title	Field study on the feasibility of MVA-MERS-S vaccination in dromedary camels
Principal Investigator	Dr. med. vet. Peter Nagy Emirates Industry for Camel Milk and Products, Dubai PD Dr. med. vet. Ulrich Wernery Central Veterinary Research Laboratory Dubai
Contribution to the One Health approach	The identical candidate vaccine MVA-MERS-S may be suitable for prophylactic immunizations of both humans and dromedary camels. Vaccination of humans may target individuals at particular risk for MERS-CoV infection such as health care workers and people in regular close contact with camels. Vaccination of dromedary camels, the natural animal host of MERS-CoV, is expected to reduce shedding of the virus from its animal reservoir and contain transmission to humans.
Abstract	**Intention and benefit for the consortium** MERS-CoV poses a major challenge as zoonotic pathogen because it naturally circulates in dromedary camels, an important livestock species and sustained source for human infection. Hypothesis is that young camels (<12 months) are the major source of virus and the important target of measures to limit camel-to-human and human-to-human transmissions. Vaccination of the animal reservoir may be most promising to prevent human infections and further spread of MERS-CoV as a pathogen of growing global concern. **Technical highlights, novelty, uniqueness** In a recent proof-of-principle experimental study in dromedary camels we demonstrated immunogenicity and efficacy of MVA-MERS-S, a Modified Vaccinia virus Ankara (MVA) vaccine expressing the MERS-CoV spike protein (Haagmans et al. 2016 Science 351:77-81). Here, we use a unique experimental setting in the United Arab Emirates to firstly investigate the feasibility and the efficacy of MVA-MERS-S immunizations in camels under field conditions and in the presence of natural infections with MERS-CoV. **Expected outcomes and perspectives** Major objectives are (i) to define the field conditions for use of the MVA-MERS-S candidate vaccine in camels, (ii) to characterize levels and longevity of immune responses elicited by MVA-MERS-S vaccination in camels (iii) to demonstrate the capacity to prevent the infection or to significantly reduce the shedding of MERS-CoV from young animals. This data may encourage camel vaccinations as successful approach to reduce the MERS-CoV zoonotic burden.

1. Working hypothesis and Research question(s)

Middle East respiratory syndrome coronavirus (MERS-CoV) infections cause an ongoing outbreak in humans fueled by multiple zoonotic MERS-CoV introductions from dromedary camels. Currently, MERS infection clusters persist in the Middle East but outbreaks also continue to occur elsewhere such as in South Korea, where a considerable fraction of the reported human cases were fatal in spite of a highly developed medical context. Primary infections in humans arise through contact with infected dromedaries and measures to prevent primary human infections need to focus on the camel-human interface. Similar to other zoonotic viruses such as avian influenza viruses, rabies virus and Hendra virus, control at the source by vaccination of the reservoir animal species may well be the method of choice to limit human acquisition of MERS-CoV. Protective experimental immunizations in adult camels has already

been proven using a Modified Vaccinia virus Ankara (MVA) vaccine expressing the MERS-CoV spike protein. However, preliminary data suggest productive MERS-CoV infection to occur primarily in young camels aged <12 months. We will therefore focus this project on the question whether MVA-MERS-S vaccination can protect juvenile camels against productive infection. Further important gaps of knowledge include (i) time periods and levels of MERS-CoV shedding in natural infections (ii) practicable routes of application, dosages and schedules for immunization, (iii) duration of vaccine induced immunity and (iv) extent of reduction of MERS-CoV shedding under field conditions.
The practicability of camel vaccinations with MVA-MERS-S will be tested in a unique setting for field studies offering an excellent experimental environment with naturally occurring MERS-CoV infections in camel calves. The studies will take place on a commercial camel dairy farm in Dubai, United Arab Emirates. The total number of dromedary camels on the farm is approximately 4,500 animals, including >300 newborn calves per year. As there is sustained transmission of MERS-CoV among the animals (100% seropositivity), the entire farm is one MERS-CoV endemic epidemiological unit. The following important research questions will be addressed:

(i) MERS-CoV-specific monitoring for seroconversion/infection in 36 dam-calf pairs spread out over the unit (year 1). This part of the study is essential to confirm the waning of maternal antibodies and average time period(s) of MERS-CoV infection in camel calves.

(ii) MVA-MERS-S prime-boost vaccination involving four experimental groups of 12 dam-calf pairs (year 2+3.) This study will address optimal application routes for practical MVA-MERS-S immunizations in the field, by performing a proof-of-principle study evaluating combined intranasal (IN) and intramuscular (IM) versus IN or IM route only prime boost applications.

(iii) MVA-MERS-S single shot immunization involving three experimental groups of 12 dam-calf pairs (year 4). Study will generate important results with regard to practicability of camel vaccination. Design will depend on the outcome of studies (i) and (ii).

2 Own previous work and publications

Our preliminary work in collaboration with partner **P1** in this consortium contributed to understanding the infection pattern of MERS-CoV in herds of dromedary camels. Important information included the observation of lower seroprevalences in juvenile camels compared to adult camels, and the development of high viral loads upon MERS-CoV infection in juveniles (1,2). The highly productive amplification of the virus in the camel population is in agreement with recent data showing an abundant DPP4 receptor expression in the upper respiratory tract of dromedary camels. Hence, infections in juvenile camels are the likely drivers of MERS-CoV transmission to humans and reduction of virus shedding from the camel reservoir is an important approach for prevention of zoonotic MERS. In the field studies outlined here, we will use an MVA-MERS-CoV candidate vaccine successfully tested for immunogenicity and protective efficacy in dromedary camels (5). This identical candidate MVA-MERS-S vector vaccine is likely to fulfill the requirements for clinical testing in humans with a first-in-man phase I/IIa study in preparation through funding provided by the German Center of Infection Research (DZIF) (3,4). Thus, the simultaneous evaluation of this vector vaccine in dromedary camels represents a genuine contribution to the One Health concept of prevention and control of diseases across species.

Five project-related publications

1. Meyer B, Müller MA, Corman VM, Reusken CB, Ritz D, Godeke GJ, Lattwein E, Kallies S, Siemens A, van Beek J, Drexler JF, Muth D, Bosch BJ, Wernery U, Koopmans MP, Wernery R, Drosten C. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014 Apr;20(4):552-9.
2. Wernery U, Corman VM, Wong EY, Tsang AK, Muth D, Lau SK, Khazanehdari K, Zirkel F, Ali M, Nagy P, Juhasz J, Wernery R, Joseph S, Syriac G, Elizabeth SK, Patteril NA, Woo PC, Drosten C. Acute Middle East respiratory syndrome coronavirus infection in livestock Dromedaries, Dubai, 2014.
*Emerg Infect Dis. 2015 Jun;21(6):1019-22.
3. Song F, Fux R, Provacia LB, Volz A, Eickmann M, Becker S, Osterhaus AD, Haagmans BL, Sutter G. Middle East respiratory syndrome coronavirus spike protein delivered by modified vaccinia virus Ankara efficiently induces virus-neutralizing antibodies. J Virol. 2013 Nov;87(21):11950-4.

4. Volz A, Kupke A, Song F, Jany S, Fux R, Shams-Eldin H, Schmidt J, Becker C, Eickmann M, Becker S, Sutter G. Protective efficacy of recombinant Modified Vaccinia virus Ankara delivering Middle East respiratory syndrome coronavirus spike glycoprotein. J Virol. 2015 Aug; 89(16):8651-6.
5. Haagmans BL, van den Brand JM, Raj VS, Volz A, Wohlsein P, Smits SL, Schipper D, Bestebroer TM, Okba N, Fux R, Bensaid A, Solanes Foz D, Kuiken T, Baumgärtner W, Segalés J, Sutter G, Osterhaus AD. An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels. Science. 2016 Jan; 351(6268):77-81

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3 Work plan including milestones

3.1 Animal studies (according to Form C)

Background and objectives:

Our main objective is to study immunization in dromedary camels under field conditions in a MERS-CoV endemic region. Dromedary camels are the natural host of MERS-CoV, the causative agent of an important zoonotic infection in humans, and young camels are probably a major source of virus transmission to humans. This project will elucidate the feasibility to induce MERS-CoV-specific immunity in juvenile camels with the objective to prevent human infection.

Methods:

Ethics statement: All animal experiments are reviewed and approved by the Animal Ethic Committee of the Central Veterinary Research Laboratory (CVRL) and the Ministry of Climate Change and Environment (MOCCAE) of the United Arab Emirates (Permit Number: 550353).

Study design: The study will involve 10 groups (n=12/group) of dromedary camel dam-calf pairs. The study will take place in a high-standard commercial camel dairy farm in Dubai (UAE). Experimental groups will be formed following the standard grouping of dam-calf pairs in the breeding process. Groups of 12 dam-calf pairs are considered optimal for animal well-being and breeding success based on long-standing experience of husbandry in this camel farm. The experimental group size (n=12) and mixed allocation of the animals from the experimental/control groups to the different breeding groups warrants thorough statistical data assessment and minimizes effects of experimental bias.

Experimental procedures: The candidate vaccine MVA-MERS-S is the identical viral vector vaccine developed for use in phase I clinical testing in humans. The application routes (intramuscular inoculations and intranasal applications) and the dosages (3x10E8 pfu MVA-MERS-S vaccine) are comparable to those used in other preclinical experiments and are in line with standard immunization procedures for dromedary camels (Haagmans et al. 2016 Science; Wernery & Kaaden, 2002). Nasal swabs are taken from mothers and calves. Serum samples are obtained through jugular vein puncture.

Experimental animals: Dromedary camels (Camelus dromedarius).

Housing and Husbandry: Animals are born and raised on a commercial camel dairy farm in the United Arab Emirates. The total number of camels on the farm is approximately 4,500. Animals are kept in open paddocks and are grouped according to the age of their calves and production stage. High standards of hygienic husbandry and biosecurity are being followed.

Sample size and allocating animals to experimental groups:

Step1: A total number of 36 dam-calf pairs (72 animals) will be used in the Step 1 experiment (allocated to three different breeding groups n=12). This distribution and sample size will allow to determine the time window of natural MERS-CoV infection and kinetics of seroconversion in this experimental setting. To address the possibility of seasonal effects in the MERS-CoV shedding from newborn camels we will monitor two groups during the peak winter calving period (December to January) and one group in the spring/summer calving period starting in March.

Step 2: A total number of 48 dam-calf pairs (96 animals) will be used in the Step 2 experiment (allocated to four different breeding groups n=12; within one breeding group 4x n=3 dam-calf pairs are allocated to the four different experimental/control groups).

Step 3: A total number of 36 dam-calf pairs (72 animals) will be used in the Step 3 experiment (allocated to three different breeding groups n=12; within one breeding group 3x n=4 dam-calf pairs are allocated to the three different experimental/control groups).

Experimental outcomes: Primary experimental outcomes for assessment are “MERS-CoV antibody titers” (IgG ELISA and microneutralization test) and “virus loads” (MERS-CoV RNA, infectious virus).

Statistical analysis: Sample sizes are calculated on the basis of the mean antibody titers obtained in previous preclinical studies with a prime-boost MVA-MERS-S immunization in mice and dromedary camels. For statistical evaluation, the camel immunization experiments will be analyzed for titers of MERS-CoV-specific antibodies and MERS-CoV-specific T cells. Effects will be further characterized by statistical classification (mean, standard deviation, median etc.).

3.2. Step 1 experiment:
MERS-CoV-specific monitoring for seroconversion/infection in dromedary camels.
This first part of the study is essential to schedule immunization in the field studies (Step 2 + Step 3). It will confirm the average time periods of maternal antibody waning and MERS-CoV infection in the camel calves. Our preliminary work suggests that MERS-CoV infection predominantly affects young, immunologically naïve animals (Meyer et al. EID, in press). Camel calves acquire maternal IgG antibodies through uptake of colostrum and high-levels of anti-MERS-CoV antibodies are found in the first week after parturition. Maternal antibodies are steadily declining within the first six months of life. Vaccinations must be applied in this time window to protect calves against MERS-CoV infection. Serum samples and nasal swabs are collected from the dams and calves on the day of parturition, week 1, week 4, then at monthly intervals. It is expected that maternal antibodies to MERS-CoV will disappear by 6 months post parturition. The major wave of natural infections with MERS-CoV is expected to occur 4-8 months after birth. We will validate an immunochromatographic assay for detection of MERS-CoV in nasal secretions at the site of the experiment (Song et al. 2015). Results will be compared against RT-PCR testing at CVRL (support by **P1**). The immunochromatographic test will be applied in the Step 2 experiment to allow for timely selection of virus infected calves for pathological examination. If sensitivity is insufficient, viral load monitoring will be done by RT-PCR instead.
Milestone 1 (project month 6): **validation of assay for rapid detection**.
Milestone 2 (**project month 12: definition of time window for immunization experiments** (criterion: time at which all animals show low residual levels of maternal antibodies and test negative for infection with MERS-CoV).
An optimal time window might be difficult to define for a large group of animals because of variabilities in antibody waning or because of seasonal patterns in the onset of infection in individual animals. In this case, immunizations with MVA-MERS-S will be scheduled earlier, e.g. at 4-8 weeks after birth and in the presence of maternal antibodies. We expect that there is no interfering impact of maternal antibodies on vaccine efficacy, based on previous experience with other MVA recombinant vaccines.

3.3. Step 2 experiment
MVA-MERS-S prime-boost vaccination in 4 experimental groups of 12 dam-calf pairs
This part of the study will address the optimization of application routes for MVA-MERS-S immunization in the field, by performing a proof-of-principle experiment comparing prime-boost applications with combined intranasal (IN) and intramuscular (IM) application, versus separate IN or IM applications, respectively. The prime-boost regimen will involve two applications of 3x10E8 pfu MVA-MERS-S in week 0 and week 8. Groups 1 to 4 (n=12 pairs/group) will each comprise 3 pairs receiving 2 x mock vaccine, 3 pairs receiving 2 x IM+IN MERS vaccine, 3 pairs receiving 2 x IM MERS vaccine, and 3 pairs receiving 2 x IN MERS vaccine. Bleedings will be done on day 0, then in 14 day intervals; 3 nasal swabs per week will be taken and samples will be stored at -80 C. Rapid diagnosis will allow for selection of three calves/group for pathological examination.
It is expected that vaccinated animals will mount **MERS-CoV specific immune responses** and longevity of the vaccine induced immune responses (antibody titers) can be demonstrated **(Milestone 3, project month 24**). Vaccinated animals will be protected from MERS-CoV infection or demonstrate significantly reduced viral shedding. Selection of a preferred immunization schedule (e.g. IM only) will be possible.
Milestone 4 (project month 36): **Demonstration of immunogenicity and protective efficacy** of a two-shot MVA-MERS-S prime-boost immunization regimen. Based on the outcome of studies up to this milestone, the choice of application regimen for project part 3 (single shot regimen) will be made.

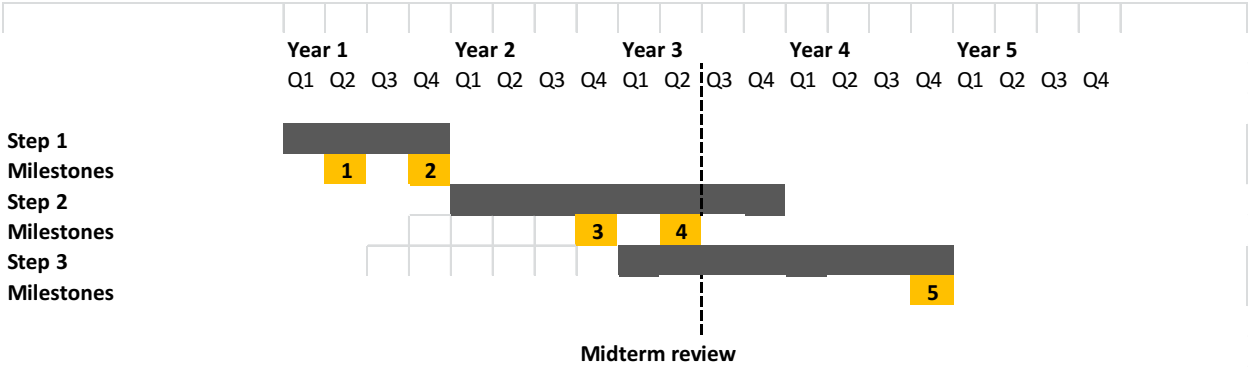
Contingency consideration at milestone 4: Immunogenicity and/or protective efficacy of the two-shot regimen might be suboptimal. In this case, we will schedule three immunizations with the MVA-MERS-S candidate vaccine for the study in Step 3. An immunization schedule with three applications would not be unusual in dromedary camels as known from the experience with other established camel vaccines.

3.4 Step 3 experiment: MVA-MERS-S single-shot immunization study

This part of the study aims at a more rapid protective immunization of camels in the field. It will involve three experimental groups of 12 dam-calf pairs. The design of the immunization schedule will depend on the outcome of studies in the Step 2 experiment. We expect that a single application of MVA-MERS-S will be sufficient for a substantial reduction of MERS-CoV excretion from juvenile dromedary camels in the first year of life. This result would be highly relevant with regard to the practical management of prophylactic vaccination in larger camel populations in the field.

Milestone 5 (project month 48): Definition of an optimal immunization schedule to be recommended for field application of MVA-MERS-S based on the results of studies in project parts Steps 2+3.

3.5 Gantt Chart:



DURC safety assessment:

The experimental work in this project has no relevance with regard to DURC.

4 Contribution to the consortium

This study is done in direct collaboration with projects **P8** (Volz/Sutter), **P9** (Herder/ Baumgärtner) and **P1** (Muth/Drosten). **P8** provides the viro-immuological follow up of the details of vaccine immune reactions. **P9** provides the pathological work up of the vaccination trial, with practical work taking place at our pathology department in Dubai (CVRL). **P1** has intiated the overall collaboration and will provide reference data as well as diagnostic methodology in particular for Step 1.

Table 1

Partner	Project area	Type of collaboration
Project 1, Muth/Drosten	Infection phenotyping	Virus detection
Project 8, Volz/Sutter	Vaccine immunogenicity and efficacy testing	Serology, T cell immune monitoring, virus testing
Project 9, Herder/Baumgärtner	Pathology of infection	Histopathology, immunohistochemistry

5 Quality assurance, standardization, data sharing

Within the Step 1 experiment we will do pilot experiments to co-ordinate and validate the serology and virus detection work across partner projects (P1, P8, P9 within the consortium).

6 Ethical and legal and considerations

All animal experiments are reviewed and approved by the Animal Ethic Committee of the Central Veterinary Research Laboratory (CVRL) and the Ministry of Climate Change and Environment (MOCCAE) of the United Arab Emirates (Permit Number: 550353).

7 Key references

Wernery U, et al., Drosten C. Acute Middle East respiratory syndrome coronavirus infection in livestock dromedaries, Dubai, 2014. Emerg Infect Dis. 2015 Jun;21(6):1019-22.
Wernery U, Kaaden O. Infectious diseases of camelids. 2nd Ed., 2002 Blackwell Science, Berlin.

Song D, et al., Al Muhairi S. Development and validation of a rapid immunochromatographic assay for detection of Middle East respiratory syndrome coronavirus antigen in dromedary camels. J Clin Microbiol. 2015;53(4):1178-82.

8 Financial summary (in Euros)

Personnel for 4 years			
Position / Salary Group	Total Budget	Duration (months)	Tasks / Justification
Personnel costs for veterinarians and veterinary technicians	297,600 US$	48	Animal handling, sample collection, laboratory work for rapid diagnosis of MERS-CoV infection

Other resources for 5 years		
Type	Total Budget	Specification / Justification
Consumables	48,000 US$	Assays for serology (ELISA) and antigen detection
Animal costs	62,400 US$	Cost for animal medication and sacrificed animals foreseen to undergo pathological examination
Equipment	--	
Travel	--	
Other	15,000 US$	Shipment of samples to Munich and Hannover

Sum: total budget:	423,000 US$ (covered by MOCCAE)
Sum: requested budget:	0
Institutional Overhead:	----
Sum: Requested Budget (50% BMBF-share for SME)	----