Teaser This review provides an overview of nonclinical in vivo models that can be used to support orphan designation in selected rare infectious diseases in Europe, with the aim to inform and stimulate the planning of nonclinical development in this area of often neglected diseases.

Nonclinical data supporting orphan medicinal product designations in the area of rare infectious diseases

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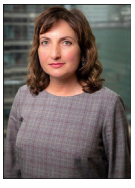

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Introduction

The European Orphan Legislation came into force in 2000, introducing a system of incentives for the development of medicines in rare diseases in the EU. COMP of the EMA is responsible for the scientific assessment of **OMPD** (see Glossary) applications. COMP evaluates the following criteria that are laid down in the EU Orphan Regulation: the rarity of the condition; the chronically debilitating or life-threatening aspects of the condition; the **medical plausibility** of the product in the condition; and the assumption of **significant benefit** over existing treatment methods [1]. At the time of initial OMPD, applicants are responsible for providing nonclinical and/or preliminary clinical evidence in support of medical plausibility. Nonclinical data are commonly used for proof of concept when considering the rarity of the orphan conditions and the possibility of submitting for OMPD at any stage of development. Thus, the quality of the submitted nonclinical evidence becomes crucial for obtaining OMPD, which ultimately unlocks

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the incentive system that can support development until marketing authorization. Challenges regarding other criteria of OMPD have been discussed elsewhere [2–4].

The Orphan Regulation resulted in a significant boost to the development of medicines for rare diseases [1]. By the end of 2018, 2134 medicines had been granted orphan status by the European Commission (EC) for a total of 524 distinct rare conditions [5]. Approximately 30% of these designations were granted at an early stage of product development, when only nonclinical proof-of-concept data were available [6]. Despite the rarity of the conditions and objective challenges of product development in such context, 164 orphan products (of which only 115 currently retain orphan status) have been authorized since the regulation came into force, with the estimated accrual rate of products in development no higher than that expected for a nonorphan product (1/10) [5,7,8].

In this review, we focus on infectious diseases, a therapeutic area that has been underrepresented in COMP analyses to date but is viewed as being particularly important. In Europe, rare infectious diseases with limited treatment options still pose a relevant threat and belong to either of two groups: diseases considered eradicated, but gravely dangerous in case of an outbreak; or diseases neglected in their endemic epidemiological regions, which occasionally occur also in Europe. Orphan regulation in this context presents an incentive to develop medicines potentially needed in the event of, for example, a terrorist attack leading to an outbreak of an eradicated disease, such as smallpox, or an incentive to develop medicines for a globally common disease, for which treatment options are limited, as is the case of tuberculosis (TB). Providing robust proof-of-concept data in a nonclinical setting can be challenging in some of these conditions.

The predictive value of all nonclinical models is limited, and one cannot underestimate the importance of clinical data in the development of a medicine. In addition, enhanced nonclinical *in vitro* techniques are currently being developed and some are already recognized by regulators as satisfactory substitutes for *in vivo* models in the nonclinical part of an application for marketing authorization. However, this review should be seen from the perspective of the regulatory committee, COMP, responsible for making an informed decision about the potential of a medicine at an early stage of development, when *in vitro* data might still be difficult to accept in the absence of preliminary clinical efficacy data. That said, COMP considers ethical and scientific arguments when assessing the nonclinical models used [9]. In this review, the COMP Non-Clinical Working Group provides guidance on the acceptability of animal models in selected infections for which designations were granted, and further comments on the shortcomings of unsuccessful applications, which can be easily avoided if the nonclinical development plan is well planned and focused on answering regulatory relevant questions.

In this review, we focus on infectious diseases considered rare within the EU, based on data from OMPD assessed by the COMP between 2000 and 2017. Non-rare infections that occur in rare diseases (e.g., infections in cystic fibrosis) were not considered, because the clinical aspects addressed in such applications were considered pertinent to the underlying rare disease. It is an analysis that is methodologically similar to a previous COMP Non-Clinical Working Group review of the nonclinical data in applications for OMPD in neurological diseases [10]. We provide a table of nonclinical **disease-relevant endpoints** (Table 1) for the purpose of OMPD. Our aim is to comment on the evidence that can be used to support future OMPDs and the proof-of-concept studies in infectious rare diseases.

Avian influenza

Avian influenza A viruses, other than H1 or H3, represent a major threat of pandemic disease, because humans lack immunity to most influenza A subtypes. Avian influenza A viruses have been divided in 'Highly' and 'Low' Pathogenic Avian Influenza viruses (HPAI and LPAI, respectively), based on their molecular characteristics and ability to cause disease and mortality in chickens in a laboratory setting. The first HPAI that infected humans in 1997 was H5N1 during a poultry outbreak in Hong Kong. Since its widespread re-emergence from 2003 to 2006, this avian virus has spread from Asia to Europe and Africa, resulting in millions of poultry infections, and several hundred cases in humans, with many human deaths. H5N1 influenza is caused by a specific viral strain subtype and, therefore, is considered to be a distinct condi-

TABLE 1

Disease-relevant endpoints accepted by COMP[a]

Pathogen	Condition	Disease-relevant endpoint
Avian influenza A Virus	Avian influenza	Survival in mice; *in vitro* neutralization activity of equine anti-H5N1 antibodies
Ebola virus	EVD	Survival, resolution of fever and clinical symptoms, reduction of viremia
VARV	Smallpox	Survival, respiratory rate, weight loss, development of secondary lesions
Cowpox virus	Smallpox, cowpox	Peak viral load, mortality
Monkeypox virus	Smallpox, monkeypox	Peak viral load, mortality
VV	Vaccinia, smallpox	Survival/weight (after a lethal viral dose); dermal lesion formation/progression; virus-specific antibody titers; cytokine levels and viremia
Bacillus anthracis	Anthrax infection	Survival
Mycobacterium tuberculosis	TB (severe, refractory)[a]	Survival, lung/spleen colony-forming unit
Fungi of the Mucoraceae	Mucormycosis	Survival
Acanthamoeba	Acanthamoeba (keratitis)[a]	Amoebic growth inhibition, infection severity grading (opacity of infected corneas)
Leishmania (*donovani* and others)	(Visceral)[a] leishmaniasis	Reduction of parasite load in liver and spleen, bone marrow, parasite clearance, immune response, physical examination in dogs
Plasmodium (*falciparum* and others)	(Severe)[a] malaria	Parasitemia clearance, mortality, likelihood of recrudescence, percentage of cure, effective dose assessment

[a] Aspect of the disease addressed in application, not highlighted in the wording of the orphan condition.

tion from common flu. For some patients, there is an unusually aggressive clinical course with rapid deterioration and high fatality rate. H5N1 influenza in humans is poorly characterized in terms of clinical endpoints. Typical systemic and respiratory symptoms include fever, chills, aches and pain, cough, and sore throat. However, avian influenza can also lead to life-threatening pneumonia and secondary bacterial infections. The incubation period ranges from 2 to 8 days and possibly up to 17 days, which is longer than for common influenza. Other avian influenza A virus subtypes are also of concern, such as the H7N7 [11] and the H7N9 viruses [12].

Five main animal species [nonhuman primates (NHPs), mice, ferrets, pigs, and cats] have been proposed and used as models of H5N1 influenza infection OMPD applications (Table 2). From a pathophysiological point of view, the best model in literature is the NHP, although its use is largely limited because of ethical concerns, the complexity of husbandry practices, and the difficulties in achieving statistical significance with the use of a reduced number of animals [13]. From a practical point of view, and considering the pros and cons, the mouse model is more appropriate and acceptable. The inflammatory effects on the respiratory apparatus are similar to those in humans. Ferrets are also considered a valid model because their pathophysiological and symptomatic characteristics are similar to those in humans. These models have all proven to be suitable to evaluate the efficacy of vaccines and antiviral drugs [14]. By contrast, guinea pig and cat animal models now are less used in drug discovery, also because of their low predictive value [14]. In some cases, rodent models (BALB/c mice) with survival as the main endpoint are still used for proof-of-concept purposes and these have been accepted so far by COMP (Table 1).

Ebola

Ebola Virus Disease (EVD) is caused by infection with a virus of the family *Filoviridae*, genus *Ebolavirus*, a family of enveloped, non-segmented negative-sense (NNS) RNA viruses. Outbreaks of Ebola happen sporadically in Africa. The 2014–2016 West Africa outbreak was from a new strain of the Zaire species (EBOV) with a reported case-fatality rate of 55%. Given this high mortality, Ebola viruses are considered Category A Bioterrorism Agents by the US Center for Disease Control (CDC) and as priority pathogens needing urgent research by the WHO. Accordingly, research with Ebola viruses is performed under Biosafety Level 4 (BSL4) conditions. The natural reservoir host of Ebola viruses has not yet been identified, but it is likely that the first patient becomes infected through contact with an infected animal, such as a fruit bat or primate. The virus can then be spread between humans through direct contact with body fluids (including blood and semen) and contaminated objects.

EVD is associated with rapid virus replication pervading most tissues and accompanied by widespread and severe focal necrosis [15]. The virus is generally detectable by PCR 48 h after infection in both lethal and nonlethal cases. However, symptoms usually occur after an incubation period of 4–10 days (or less commonly between 2–21 days). After a sudden onset of 'flu-like' symptoms (fever, myalgia, and chills), and vomiting and diarrhea, the disease can rapidly evolve into a severe state with a rapid clinical decline and death due to shock, hemorrhage, and multiorgan failure.

TABLE 2

Models of avian influenza infection[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
NHP: Cynomolgus macaque	Infection with multiple H5N1 strains inoculated through different routes	Respiratory tract is major target; acute respiratory distress syndrome, fever, nasal discharge, sneezing, lethargy, weight loss	Similar symptomatology to human	Different pathophysiology; ethical concerns	[85,86]
Mouse: *BALB/c* and *C57bl/6*	Infection with multiple H5N1 strains inoculated through different routes	Pulmonary infection, with systemic spread; weight loss, huddling, ruffled fur, lethargy, hypothermia; high lethality	Similar pathogenesis to human; easier for statistical analysis and husbandry	Different clinical symptoms and signs than in humans	[87,88]
Ferret	Infection with multiple H5N1 strains inoculated through intranasal route	Virus replication in upper and lower respiratory tract and in multiple organs including brain. Severe lethargy, fever, weight loss, transient lymphopenia	Similar pathophysiology and symptoms to human	Inoculation routes other than intranasal pose difficulties. Paucity of laboratory reagents	[89,90]
Guinea pig	Infection with multiple H5N1 strains inoculated through intranasal route	Virus replicates in respiratory tract but not in other tissues. Tracheobronchitis, bronchointerstitial pneumonia, nasal discharge, cough, labored breathing, fever, weight loss	Similar pathophysiology to human	Paucity of laboratory reagents; lacks many of clinical signs seen in humans	[91,92]
Cat	Infection with H5N1 strains inoculated through intratracheal route or feeding on virus-infected chicks	Virus detected in respiratory, digestive tract, nervous, cardiovascular, urinary, lymphoid, and endocrine tissue; fever, conjunctivitis, lethargy, labored breathing	Pattern of H5N1 virus attachment to lower respiratory tract cells closely mimics that observed in humans	Ethical concerns, complex husbandry and low availability	[93]

[a] Take-home message: mouse model is acceptable to support the orphan designation because of the similar pathogenic mechanisms, accessibility, and easy-to-reach statistical significance.

The gold standard nonclinical model for EVD is the infection of NHPs, especially cynomolgus or rhesus macaques (favored NHP models). As already mentioned, NHP studies are expensive and limited for ethical reasons, and they are usually performed at late nonclinical stage, once proof of concept has been obtained in smaller models. The nonclinical endpoints accepted so far by COMP are similar to the known clinical endpoints. This is because of the similarities of the clinical features of EVD in humans to those observed in nonclinical models (Table 3).

Five products have obtained OMPD for the treatment of EVD and all designations to date have been based on nonclinical data. Whenever studies in NHP models were not available, mouse models were considered acceptable when methodology and results were robust. Among small animals, mouse models show rapid onset of viremia and high viral burden in the spleen, liver, and multiple organ tissues. Lymphopenia, thrombocytopenia, kidney dysfunction, and liver damage are also observed. However immunocompetent mice are resistant to wild-type EBOV (WT EBOV); thus, mouse-adapted EBOV is needed for the infection to occur. By contrast, WT EBOV is lethal to suckling mice and immunodeficient mice (e.g., SCID mice), which lack functional B and T cell responses. Therefore, it is possible to challenge mice with a mouse-adapted Mayinga EBOV strain. In some cases, studies in NHP models were also available and used to support medical plausibility.

Orthopoxvirus infections

Currently, ten species of virus are included in the genus *Orthopoxvirus*, which belongs to the *Poxviridae* family. For regulatory purposes, each individual pathogen is considered to cause a separate orphan condition (e.g., smallpox infection, monkeypox infection, etc.) and, thus, individual applications for each virus are generally required.

Smallpox and vaccinia are caused by *Variola virus* (VARV) and *Vaccinia virus* (VV), respectively. Smallpox was declared eradicated in 1980 because of successful prophylactic vaccination during the 20th century [16]. Replication of VARV occurs in the cytoplasm and infected macrophages carry the virus to the lymph nodes. Consequently, small vessels of the dermis become infected, resulting in the typical skin pustules [17]. Clinical forms of smallpox can be divided into five varieties: ordinary, modified, variola sine eruptione, flat, and hemorrhagic. Cytopathic effects of the virus can lead to death, although the cause of death remains controversial, because multiple mechanisms are involved. Mortality of smallpox was ~30%, killing ~500 million people over the past 100

TABLE 3

Models of Ebola virus infection and/or disease[a]

Animal model	Method of generation	Features of model	Advantages of model	Disadvantages of model	Refs
NHPs (Cynomolgus macaque is preferred)	Experimental infection	Fever, anorexia, rash, increase in liver enzymes and disruption of coagulation	EBOV infection in NHP recapitulates human disease in clinical symptoms, and histopathology; Cynomolgus macaques can be used for vaccine studies and Rhesus macaques for postexposure studies	Ethical concerns, complex husbandry requirements and low availability; limited space in BSL4 facilities	[94–97]
Mouse: collaborative cross mouse	Infections in cross of eight inbred founder mouse strains (C57BL/6 J, A/J, 129S1/SvlmJ, NOD/ ShiLtJ, NZO/H1LtJ) and three wild-derived strains	Rapid onset of viremia and high viral burden in spleen, liver, and other organs; lymphopenia, thrombocytopenia, kidney dysfunction, liver damage	Useful for screening for new compounds	Model presents with only a few features of human disease; no consensus on validity of any existing Ebola infection mouse models, which makes it difficult to interpret data;	[98]
Mouse: humanized mouse model NOD/ShiLtJ background	Infections in NOD/ShiLtJ strain	Defects in antigen presentation, T lymphocyte repertoire, natural killer cell function, macrophage cytokine production, wound healing, C5 complement	Common choice for Ebola in immunodeficiency	immunocompetent mice are resistant to WT EBOV. In mice infected with mouse-adapted EBOV, no coagulopathy is observed	[99]
Syrian hamster	Experimental infection	Fever, anorexia, and dehydration; drop in platelet count, increased fibrin deposition and prolonged prothrombin and partial thromboplastin time	Infection with mouse-adapted-EBOV is similar to that in humans, including, severity of coagulopathy, which does not occur in mouse and guinea pig. Pathology of spleen and liver similar to human	Limited availability of hamster-specific laboratory reagents	[100]
Duncan–Hartley and Strain 13 guinea pigs	Experimental infection	Virus detected in lymph node macrophages 24 h after inoculation, spreads to spleen and liver thereafter, and subsequently to other organs and tissues	Allows larger sampling sizes; histopathology similar to mice, NHPs and human; can be used for antibody therapy testing	Strain 13 shows altered immune response, not fully representative of human Ebola	[100]

[a] Take-home message: generally, the model of choice to study medical plausibility in Ebola would be NHP despite all the ethical considerations. However, for an orphan designation, the COMP would also accept data generated in small model organisms, such as mice.

years. A single case of smallpox anywhere in the world would be a global health emergency [18].

No animal reservoirs exist in nature, and most animal species cannot be infected even in the laboratory [19]. Smallpox is also challenging to study because of biosafety restrictions. Therefore, **surrogate disease models** are needed. In this context, to facilitate drug development when circumstances do not allow proper clinical evaluation, the US Food and Drug Administration (FDA) issued the 'Animal Rule', which states that efficacy data can be obtained from appropriate animal models and bridged to humans [20]. Indeed, the research of smallpox treatment is ongoing as evidenced by a recent (2018) FDA first approval of a drug for this pathogen, tecovirimat [18]. Interestingly, in Europe, tecovirimat has been granted an OMPD for the treatment of cowpox, but so far no further regulatory steps have been taken towards marketing authorization in Europe.

One application exploring the efficacy of the drug in rabbits infected with a surrogate *Orthopoxvirus*, rabbitpox, was presented to COMP [21]. The advantage of the rabbitpox model is the ability to produce a natural aerosol transmission of the virus between animals with secondary lesions, although the rabbitpox infection does not occur in humans. Other models comprising *Orthopoxvirus* in the literature include VV, ectromelia, cowpox, and monkeypox virus in mice [22]. Interestingly, besides VV, monkeypox and cowpox [23] also infect humans. Although mice models can be used in studies that are practical and can reach statistical power, the differences in immune system responses between mice and humans can result in differences in epitope recognition, thus hampering translatability to humans [22]. Notably, the Ind-3a strain of VARV was explored by the Institute of Cancer Research (ICR) in SCID mice [24].

Monkeypox virus was additionally used to infect monkeys [25], squirrels [26], prairie dogs [27], and pigs. Moreover, this virus was administered via the intranasal inoculation route to mimic natural infection in hamsters, rabbits [28], rats [29,30], and mice [28,31–35]. However, because the human infectious monkeypox dose is unknown, it is hard to establish the translational benefit of these models [36].

The cowpox virus, another member of *Poxviridae*, shares homology with monkeypox and VARV [37], which allows the use of this virus as a model to study smallpox [38]. Importantly, in contrast to VARV, both cowpox and monkeypox virus require a lesser biosafety 2 level to work with.

The authentic VARV is able to infect cynomolgus monkeys, specifically the Harper and India 7124 strains. The model is characterized by systemic disease with features of human smallpox with a high lethality [39].

Overall, the ideal model would have the characteristics of a generalized dissemination with secondary lesions, in animal-to-animal spread, and high lethality. Given that no animal model perfectly mimics smallpox in humans, it is considered more suitable to test the efficacy in several animal models to increase the translatability to human smallpox. However, with regard to the OMPD, data produced in one animal model would be acceptable.

VV infects not only the reservoir species (most likely candidates are sylvatic rodents), but also humans, resulting in a skin infection. In a generalized infection, virus spread is thought to occur through the regional lymphatics to the bloodstream, resulting in primary viremia [40]. Clinical signs of generalized vaccinia include a diffuse erythematous maculopapular rash scattered over the body; papules become vesicles and generally heal over within 15 days, leaving a typical scar in the skin of people and animals affected [41]. Clinical characteristics of vaccinia complications, especially in immunocompromised patients, include eczema vaccinatum in patients with a history of eczema or atopic dermatitis, persistent infection with tissue necrosis (vaccinia necrosum), postvaccinal encephalitis, myocarditis, and ischemic cardiac events [42–44]. Vaccinia vaccination is necessary after a smallpox outbreak or after a bioterrorist attack (Table 4).

For VV infection, there are several available rodent models in which disease-relevant endpoints can be measured (Table 4). These models mimic certain aspects of the disease (e.g., progressive cutaneous infection in an immunocompromised host) and can be considered valuable to study how VV modulates the host immune response. However, unlike the intranasal route of infection, intradermal inoculation is localized, without generalized clinical signs of illness similar to those observed during intranasal infections (e.g., weight loss) [45]. For the purposes of OMPD, presentation of data in one valid animal model would be sufficient.

Anthrax

Bacillus anthracis (*B. anthracis*) is a Gram-positive, rod-shaped bacterium. Human infection can be naturally acquired from contact with infected grazing animals that have ingested soil contaminated with *B. anthracis* spores, or from occupational exposure to infected and/or contaminated animal products. This type of infection is usually cutaneous or, less frequently, gastrointestinal.

The inhaled forms of the infection are usually accidental or related to bioterrorism, although they often occurred previously in industrial settings, such as while working with wool. *B. anthracis* spores germinate within the alveolar lung macrophages and produce anthrax toxin, responsible for triggering the cascade of inflammatory events and starting the clinical manifestations of the disease. The incubation period of inhaled anthrax typically lasts 2–10 days, and the first symptoms are flu-like, followed by a rapidly progressive phase of systemic manifestations culminating over the course of 12–24 h in the development of bacteremia and rapid clinical deterioration with high fever, dyspnea, and shock, with 100% death rates. Cutaneous forms are the mildest, often self-limited, with mortality of ~20% if untreated. The available treatments for anthrax infection comprise antibiotics. Still, survival is poor at 50–60% in nonclinical models, which provide the only indicative data of **disease-relevant activity** because the clinical experience in inhalation anthrax is limited. All the OMPDs granted so far target the inhalation form of anthrax infection.

Selecting animal model for studies on anthrax might be complex, because it dependents on many interrelated factors, such as the specific aim of the research, the differing attributes of the animal species, and the manner and route of exposure. The best animal models developed for the evaluation of anthrax countermeasures are NHP and rabbit models. However, limitations, such as costs, ethical issues, housing and maintenance constraints, restrict their use for the final evaluation of medicinal products, just before licensure for human use [46]. During the initial steps of

TABLE 4

Models of *Orthopoxvirus* infection[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Models of VARV infections					
Rabbit: New Zealand white rabbits – rabbitpox; Vaccinia (WR strain)	Intradermal infection through thighs.	Disseminated lethal disease at very low inoculum with secondary lesions, respiratory involvement, and natural aerosol transmission between animals	Genetic similarity to VARV, similar pathophysiology to human disease, low dose of virus leads to lethal disease, correlation of viral burden with disease progression, model of disease with lesions	Short incubation time with short survival after infection	[18,101]
Mouse: variola	Intranasal challenge with strain Ind-3a of VARV in immunocompetent ICR mice and immunodeficient SCID mice	Infection restricted to respiratory organs, not progressing to second and third stages	Prophylactic research, high susceptibility to virus; great similarity regarding inflammatory destructive effect on respiratory tract organs	Asymptomatic infection; infection limited to brain and respiratory tract; not suitable for study of therapeutic treatments	[22]
Mouse: vaccinia BALB/c, C56BL/6	Intraperitoneal, intranasal infection	Challenge with virus induces cellular and humoral immune responses	Statistical power, practicality of model	Lack of obvious vascular involvement; lethal infection requires a substantial viral inoculum; pathology of advanced disease differs from smallpox in human	[22]
Mouse: ectromelia (mousepox)	Intranasal infection, application through scars on tail	Infections at low virus doses, transmittable between mice; disease severity dependent on mouse, virus strain, and route of infection	Statistical power, practicality of the model; natural pathogen of mice	Rapid mortality precludes development of lesions	[22]
Mouse: cowpox	Intradermal, intratracheal	Disease course analogous to smallpox in humans	Virus highly virulent for mice; human is a natural host	Large viral inoculum needed to obtain lethal infection; pathology of advanced disease differs from smallpox in human	[22]
Mouse: monkeypox	Intranasal, intratracheal, intraperitoneal	Systemic disease: virus detected in multiple organs, including lungs and kidneys; pustule lesions	Human is a natural host	Absence of rash	[32,36]
Rat: vaccinia	Intradermal, intravenous	Bioluminescent imaging in live organism	Statistical power, practicality of model	Absence of primary lesions	[102]
Monkey: monkeypox	Intravenous, intratracheal	Necrotizing lesions at all affected sites, including lungs, lymph nodes, thymus, spleen, skin, oral mucosa, gastrointestinal tract, and reproductive system	More severe disease in NHPs; good surrogate for human smallpox	Limitations in group size and ethical considerations	[22]
Monkey: variola	Intravenous, aerosol	Uniform acute lethality when inoculated intravenously in high doses; lower doses result in less fulminant, systemic disease and lower mortality	Mimics incubation and prodromal phases of human smallpox by creating instantaneous viremia and systemic spread of virus to target tissues	Less susceptible to virus than humans; ethical concerns, complex husbandry, limited availability	[22]
African dormouse: monkeypox	Intranasal	Replication in nasal mucosa causing necrosis and hemorrhage with systemic spread to lymph nodes, spleen, liver, and other tissues, causing severe necrosis and/or hemorrhage leading to death	Many of histopathological features similar to those in smallpox-infected humans	Not specifically noted in consulted literature	[36]
Squirrel: monkeypox	Intranasal, oral, scarification routes	Airborne and direct contact transmission to healthy animals	High susceptibility to virus challenge	No skin lesions	[36]

TABLE 4 (*Continued*)

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Prairie dog: monkeypox	Intranasal, intraperitoneal, scarification route	Congo Basin and West African clade used; necrotizing bronchopneumonia, conjunctivitis, and tongue ulceration	High susceptibility to virus challenge	Not specifically noted in consulted literature	[36]
Models of VV infection					
Mouse: C57BL/6	Intradermal inoculation in left ear dorsal pinna	Viral pathogenicity assessed based on lesion formation and size	Resembles scarification route used during smallpox vaccination; valuable model to study how VV modulates host immune response	Localized infection, no generalized clinical signs of illness like those observed during intranasal infections	[45]
	Intranasal inoculation	Model of protective vaccination based on infected mice maintaining weight	Disease-relevant endpoint (weight maintenance)	No skin lesions	
Mouse: BALB/c	Intraperitoneal	Mice develop virus-specific neutralizing antibodies, immune responses can be measured	Can be used in other *Orthopoxvirus* diseases; good for studying mechanism of poxvirus-induced innate and adaptive immune response	No skin lesions	[103]
	Orally ingested contaminated VV milk	Generalized infection; virus-specific neutralizing antibody titers and viral DNA titers in fecal, blood, and tissue samples	Pathogenesis and distribution of virus through all organs; virus-specific neutralizing antibody titers as disease-relevant endpoint	No skin lesions	
	Intranasal	Reduced survival; acquired protective immunity can be tested with a secondary lethal dose of virus and assessed measuring weight, virus-specific antibody titers and cytokine production in lungs and spleen	Good for studying acquired protective immunity; virus-specific neutralizing antibody titers as disease-relevant endpoint	No skin lesions	
Mouse: Nc/Nga	Intranasal inoculation	Atopic mouse model sensitized with ovalbumin to induce spontaneous skin lesions and elevated serum levels of immunoglobulin (Ig)E; mice develop atopic dermatitis	Similar to human atopic dermatitis; pathology of eczema vaccinatum can be studied	No skin lesions	[104]
Rabbit: New Zealand	Intranasal inoculation of Bovine Herpesvirus 1 Glycoprotein D (BoHV-1)	Mice develop virus-specific neutralizing antibodies, immune responses can be measured	Mechanism of poxvirus-induced immune response can be studied; well-established model to study pathogenesis and natural route of BoHV-1 infection, and efficacy of cattle vaccines	No skin lesions	[105]
Rat: Rag2$^{-/-}$	Intradermal inoculation in immune-deficient females	Viral load/clearance rate in tissues in published study was monitored using luciferase bioluminescence assays	Suitable visualization model that recapitulates infectious and clinical features of human smallpox in immunodeficient populations	Not specifically noted in consulted literature	[102]
Mouse *Ifngr1*^{tm1} knockout	Mice homozygous for *Ifngr1*^{tm1} knockout mutation	Mice have normal T cell responses but are defective in natural resistance	Increased susceptibility to VV infection	Not specifically noted in consulted literature	[99]
Mouse *Mb21d1* (cGAS) knockout mice	Mice homozygous for *Mb21d1* knockout mutation	Homozygotes challenged with VV exhibit higher viral titers and mortality	Increased susceptibility to DNA viruses (VV and West Nile virus infection); useful in studies of response to viral infection or cytosolic DNA	Not specifically noted in consulted literature	[99]

[a] Take-home message: various *Orthopoxvirus* models can and are being used interchangeably to explore treatment efficacies in smallpox, vaccinia, monkeypox, and cowpox. Mouse models for VV infection mimic clinically relevant endpoints of vaccinia and were considered valuable by COMP to support orphan designation because of their predictive value, based on a similar pathophysiology. Adequate data produced in one animal model are sufficient for orphan drug designation purposes.

TABLE 5

Models of anthrax[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
NHPs: Rhesus macaque, Cynomolgus macaque, African green monkey	Aerosolized B. *anthracis*, intranasal, intubation, *trans*-tracheal	Onset of disease 3–25 days post challenge; can die acutely with no clinical sign of disease; edema, hemorrhage, and necrosis in lungs; damage to mediastinum, meninges, adrenal glands, gastrointestinal tract, and urogenital organs	Fully mimics human infection; develops anthrax meningitis; similar PK/PD parameters as in human; can be used to estimate human administrations and dosages; good as a vaccine model	Ethical considerations, supply limitations, complex maintenance	[106–112]
Rabbit: New Zealand White rabbit, Dutch belted rabbit	Challenge with fully virulent strains of *B. anthracis*, subcutaneous injection, intranasal, or aerosol exposure; most often aerosol in a muzzle-only mask	Susceptible to lethal infection; clinically less severe than in NHPs and humans; no occurrence of meningitis.	Pathology similar to humans regardless of infection route; clinical similarities with human anthrax, especially cardiac involvement; accepted vaccine and therapeutic drug model	Lack of a clear dose response; different to human anthrax (i.e., significant CNS involvement), more acute disease in rabbits; innate sensitivity to some antibiotics, limits use in therapy studies	[113]
Guinea pig	*B. anthracis*/spore challenge: resistant to toxin	Different symptoms from human (no fever)	Good for studying pathogenesis of inhalational anthrax; allows collection of adequate amounts of tissues for analyses, and small enough to allow adequate cohort sizes	Performs poorly with 'cell-free' or subunit vaccines because of limited efficacy; unique pathogenesis different from human	[110,114–116]
Mouse: various strains; immunocompetent C5 mice (e.g., C57Bl/5)	Virulent toxin challenge	Time to death after challenge: 3.3 days; acute disease characterized by extensive edema and large titer of bacilli in blood and organs	BALB/c survive longer, enabling medicine testing. A/J mice die quickly after 2 days; attenuated *B. anthracis* can be studied under biosafety level 2 conditions	Not adequate to test aluminium adjuvanted; protective antigen vaccines; pathology does not represent human condition	[114,117–120]
Mouse: immunodeficient (C5)	Attenuated strains of *B. anthracis* challenge	Death after low doses	Useful for studying role of toxins in pathogenesis and for preliminary efficacy of vaccines and therapeutics	Not specifically noted in consulted literature	[110]
Rat	Toxin challenge	Relatively resistant to parenteral challenge with spores, but extremely sensitive to injected anthrax toxin	Sensitive model for efficacy of antitoxin medicines	Not an ideal model for whole *B. anthrax*/spore challenge	[118,121]

[a] Take-home message: COMP would find the rabbit model most appropriate to study medical plausibility, and the addition of NHP data could support authorization of the product based on nonclinical data only.

drug development, small mammals, mainly mice, guinea pigs, and rats, have been used to study the pathogenesis, treatment, and prevention of anthrax (Table 5).

The products that received OMPD include several monoclonal antibodies directed against the anthrax toxin, which have shown significant improvement of survival in nonclinical models, alone and when administered in combination with antibiotics. The designated products were tested in rabbits and/or NHP (Table 5). Both NHPs and rabbits have been accepted by the COMP as models for the development of new medicines and vaccines for anthrax. These models were also accepted by FDA as valid models in the authorization of anthrax products under the animal rule [46]. For treatment purposes, the candidate products are usually administered upon detection of significant increase in body temperature and/or anthrax protective antigen (PA) in the serum, which is considered by the COMP as a valid approach.

Tuberculosis

Mycobacterium tuberculosis infects mainly lungs, but can spread to other organs, producing extrapulmonary TB. Infection occurs upon inhalation, when the infectious droplets settle in the airways, predominantly in the upper part of the respiratory tract. The immune system responds through macrophages that present mycobacterial antigens to T cells. Macrophages then envelop the bacteria, forming granulomas, where it continues to reproduce, eventually killing the immune cell and producing solid necrosis [47].

Applications for products intended for TB treatment have been presented several times to COMP. From the ten applications submitted, only one represented a vaccine, whereas the rest were intended for already infected individuals. All applications presented data with a nonclinical mouse model of the infection, while one was complemented with additional guinea pig model data. Additionally, TB models utilizing the New Zealand rabbit, Cynomolgus macaque, Chinese tree shrew, Wistar rat, castrated male Friesian-cross calf, and zebrafish larvae or adult zebrafish are known (Table 6) [48].

The mouse TB model is characterized by homogeneous pathological changes and bacterial burden, which makes it an appropriate model for rapid anti-TB chemical drug evaluation. However, mice have a varied length of latent period, with high bacterial

TABLE 6

Models of TB[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Mouse	Aerosol or tail vein infection	Relatively low susceptibility to bacteria	Appropriate model to study vaccines, drugs, immune mechanisms, and host genetics	Missing obvious clinical expression of infection, different TB granuloma structure compared with human; without disseminated disease, interindividual variation in infection	[48,122]
Rat	Aerosol route infection	Lower susceptibility to bacteria than mice	Ideal model to study biological characteristics and formation of pulmonary granuloma	Different TB granuloma structure compared with human; pulmonary bacterial load in latent infection is relatively high	[48]
Guinea Pig	Aerosol or subcutaneous infection	High susceptibility to bacteria	Replicates many aspects of TB infection in humans; ideal to test vaccine efficacy	Paucity of specific immune reagents; general clinical features of TB are missing, no spontaneous latent infection	[48,122]
Rabbit	Aerosol infection with *Mycobacterium bovis*	Very low susceptibility to *Mycobacterium tuberculosis*; *M. bovis* is more often used	*M. bovis* infection resembles human pulmonary pathology; used to test pathogenesis of disease	Missing obvious clinical expression of infection, missing specific immune reagents; *M. bovis* pathogenesis is different from that of *M. tuberculosis*	[48,122]
NHPs	Aerosol infection	High susceptibility to bacteria	Ideal for testing pathogenesis and vaccine or drug development	Interindividual variation, paucity of specific immune reagents	[49,122]
Zebrafish (adult and embryonic larvae)	Infected with *Mycobacterium marinum* via various inoculation routes	No susceptibility to *M. tuberculosis*, but to *M. marinum*. Fish develop necrotic granulomas	*M. marinum* infection with necrotic granulomas resembles human infection; used for pathogenesis research and screening of therapeutics	For infection with *M. marinum*, clinical manifestations and symptoms of TB are missing, absence of specific immune reagents	[48,50]

[a] Take-home message: from the experience of COMP, it can be concluded that the combined use of mice and guinea pig models should be pursued, because their characteristics complement each other and produce robust data. The Cynomolgus macaque model would be most appropriate because of its similarity to human, but is not required by the COMP because of ethical reasons.

burdens and heterogeneous starting time points [48]. The guinea pig model is susceptible to the infection and presents with similar symptoms and pathophysiology as humans. Thus, this model is appropriate for the evaluation of vaccines. By contrast, the guinea pig seldom presents liquefaction and cavitation of pulmonary granulomas and does not exhibit a latent form of infection [49]. Recently a zebrafish model, an unusual organism for TB because it does not have lungs, emerged. This model is valuable for visualization of early steps of TB pathogenesis [50] and, as such, is ideal for the initial identification of new antimycobacterial drugs. However, the zebrafish model, if submitted as sole evidence, would not be enough to substantiate the proof of concept of the product because of its limitations in reproducing the clinical aspects of TB. In cases where this model was used as part of a larger nonclinical development, it would be assessed as supportive evidence on a case-by-case basis (Fig. 1).

Mucormycosis

Mucormycosis refers to fungal infections caused by species of the family Mucoraceae, which are members of the order of Mucorales, Subphylum Mucoromycotina [51]. The most common species isolated from patients include *Rhizopus*, *Mucor*, and *Lichtheimia*. These pathogens are ubiquitous in nature and infection is usually seen in patients who are immunocompromised. In developed countries, cases are mostly seen in transplant recipients and patients with hematological malignancies, whereas, in developing countries, the infection occurs mostly in patients with diabetes mellitus [51]. The infection is characterized by angio-invasion resulting in thrombotic and infarcted lesions in the affected tissues. Based on its clinical presentation and anatomic site, mucormycosis is classified into six major clinical forms: rhinocerebral; pulmonary; cutaneous; gastrointestinal; disseminated; and uncommon rare forms, such as endocarditis, osteomyelitis, peritonitis, and renal infection [52].

Several *in vivo* models have been discussed in the literature using a plethora of fungal strains (Table 7). Most common references include neutropenic rodent models infected via different routes, as, for example, intravenously to generate a disseminated infection [53] or intratracheally [54] for the production of a pulmonary phenotype. In those settings, cyclophosphamide or cytarabine can be used for inducing neutropenia [53,54]. Study endpoints have included not only survival, but also residual fungal burden and other endpoints, such as pulmonary infarct scores [54]. Diabetic mouse models, where diabetes is induced by streptozocin, have also been used to study mucormycosis [55]. Intranasal challenge of diabetic mice with Mucoraceae spores results in specific enhanced susceptibility similar to humans with diabetes. Nonlethal murine models of cutaneous mucormycosis [56], as well as nonrodent models [57] have also been discussed.

So far, COMP has granted one successful designation for the treatment of mucormycosis. The application included, among others, nonclinical data in a neutropenic mouse model challenged intratracheally with a strain of *Rhizopus oryzae*. Neutropenia was induced by cyclophosphamide and cortisone, and mice were infected intratracheally and then treated with the study drug starting 8 h after infection for a total of 5 days. *In vivo* efficacy

TABLE 7

Models of mucormycosis[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Mouse	OF1 mice immuno-compromised 1 day before infection with cyclophosphamide and 5-fluorouracil; challenged intravenously with *Mucor circinelloides*	Produces acute invasive infection, with animals dying within days after challenge	Allows for survival, tissue burden, and histopathological studies in various tissues	Severe model that might not recapitulate nondisseminated infection	[33]
Rabbit	New Zealand white rabbits treated with cytarabine 3 days before challenge; endotracheal inoculation with sporangiospores of various species (*Cunninghamella bertholletiae, Rhizopus oryzae, M. circinelloides* or *Rhizopus microsporus*)	Development of fungal pulmonary infarcts	Recapitulates pulmonary mucormycosis in neutropenic hosts	Might not recapitulate nonpulmonary routes of infection	[54]
Mouse	Immunocompromised dBALB/c mice; subcutaneous injection of *R. oryzae* conidial suspension	Cutaneous and soft tissue infection	Recapitulates features of cutaneous aspergillosis	Produces nonlethal phenotype	[56]
Mouse	OF1 diabetic mice injected with *C. bertholletiae* into lateral tail vein	Produces acute invasive infection, with animals dying within days after challenge	Infection studied in a diabetic setting	Questionable relevance for nondiabetic settings	[55]
Zebrafish	WT AB zebrafish larvae infected with *M. circinelloides*; pathogen injected into hindbrain ventricle (another model using swim bladder infection also possible)	Filamentous fungal growth in hindbrain, reaching into forebrain and invading ventral muscular tissue	Allows real-time microscopy analysis of early innate immune response; mimics range of aspects of human disease	Limited reproducibility of disease features in humans; limited value in drug efficacy testing	[57]

[a] Take-home message: COMP would accept data in mice as supportive for potential efficacy. Infection in mice via the intravenous or intrathecal route allows multiple endpoints to be studied.

was assessed by comparing the survival time of the active and placebo groups of mice. From the experience of COMP, it can be concluded that such mucormycosis models in immunocompromised animals that recapitulate aspects of the human disease could be useful in supporting OMPD applications.

Acanthamoeba keratitis

Acanthamoeba are a genus of amphigoric amoebae that is widely distributed in the environment, being present in the air, soil, and water. *Acanthamoeba* spp. cysts are capable of enduring extreme environmental conditions [58].

Acanthamoeba has two developmental stages: cysts and trophozoites. Although trophozoites are the infective forms, both can enter the host through the eye, the lower respiratory tract, or ulcerated or broken skin. When *Acanthamoeba* spp. adhere to the eye surface, it can result in keratitis in otherwise healthy individuals, particularly contact lens users. If the parasite invades the host through the respiratory system or broken skin, it can access the central nervous system (CNS), causing granulomatous amoebic encephalitis (GAE), disseminated disease, or skin lesions in individuals with compromised immune systems.

The main risk factor for acanthamoeba keratitis (AK) is the use of contact lenses and corneal trauma [59–61]. AK infection can result in radial neuritis and severe pain [62], eyelid ptosis, conjunctival hyperemia, and epithelial ulcers [62], often followed at later stages by the appearance of a ring-like stromal infiltrate [62,63]. AK can progress to scleritis and, in severe cases, ocular enucleation [64]. The pathophysiology of this infection involves sequential events that includes the production of several pathogenic proteases that degrade basement membranes and induce the cytolysis and apoptosis of the cellular elements of the cornea, culminating in dissolution of the collagenous corneal stroma [65].

Available models used for AK assessment include *in vitro* and *in vivo* models. *In vitro* axenic models assess the killing kinetics of potential treatments against excysted trophozoites. *In vivo* models published include mouse, rat, Chinese hamster, rabbit, and pig models (Table 8). None of the models available fully recapitulates the disease in humans. Rats and mice developed similar clinical responses following successful infection after corneal scratching, scratching followed by corneal cover with contact lens, and intrastromal injection. The latter route has shown the highest infection success rate in rodents [66]. Mice are more infection sensitive, with higher animal mortality [66]. Although intrastromal injection results in endophthalmitis, this procedure does not mirror the natural human infection route. However, the intrastromal injection model still has innate value for the study of the immunological response to *Acanthamoeba* spp. infection [67].

In contrast to other animal species, *Acanthamoeba* readily adheres *in vitro* to corneas of pigs, Chinese hamsters, and humans [68,69]. The pig model allowed the evaluation of infections with *Acanthamoeba* spp. through application of human contact lenses [66]. However, unlike the persistent nature of human AK infection, a spontaneous resolution of the disease usually occurs in pigs. The Chinese hamster model of infection closely resembles acute-phase infection in humans [70], but has no nonacute phase and is also self-limiting [59]. In the rabbit model, infection of the eye led to necrosis and inflammatory response. The above-described animal models are considered relevant despite the identified weaknesses; however, there remains a lack of a comprehensive model that fully encompasses the pathology of progressive AK in humans. Previously, COMP has reached positive opinions for initial OMPDs applications where nonclinical justification was based solely on observations *in vitro*. However, it is considered a matter of exception and normally *in vivo* data would be needed to support the assumption of medical plausibility.

TABLE 8

Models of AK[a]

Animal model	Methods of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Mouse	Intrastromal seeding of amoebas; scratching of cornea + one-time challenge with pathogen; scratching of cornea + continuous challenge with pathogen through contact lens application	*Acanthamoeba* spp. trophozoites do not bind to murine cornea; either debridement in combination with sufficient pathogen challenge time or pathogen injection is necessary for infection	Availability of mice in large numbers allowing statistical analysis; existence of an array of laboratory reagents	Optimal route of infection does not represent normal route of infection in human; animals might develop concomitant endophthalmitis; spontaneous remission or lethality can happen	[123]
Rat	Intrastromal seeding of amoebas; scratching of cornea + one-time challenge with pathogen; scratching of cornea + continuous challenge with pathogen through contact lens application	*Acanthamoeba* spp. trophozoites generally do not bind to rat cornea; either debridement in combination with sufficient pathogen challenge time or pathogen injection is necessary for infection	Availability of rats in large numbers allowing statistical analysis	Optimal route of infection does not represent normal route of infection in human; animals can develop concomitant endophthalmitis; spontaneous remission can occur	[123,124]
Hamster: Chinese hamster, Syrian hamster	Seeding through contact lenses after corneal debridement; intrastromal injection	*Acanthamoeba* spp. trophozoites do not adhere to cornea (see above); corneal lesions induced by live amoeba resemble those in infected human eyes	Ideal model to study pathogenesis and immunological response to *Acanthamoeba* corneal infection	Only acute infection occurs; infected animals show spontaneous remission, which does not occur in humans	[124]
Rabbit	Seeding through contact lenses after corneal debridement; intrastromal injection; microinjection anterior to cornea	Trophozoites rarely bind naturally to rabbit cornea and, thus, either debridement before pathogen challenge or pathogen injection is generally necessary for successful infection to occur	Histopathological similarity to human disease; high histological similarity with human cornea	Infection rates can be as low as 50%; infection is self-limiting, unlike in humans; intrastromal injection can cause endophthalmitis and mortality; microinjection, as opposed to intrastromal injection, has more pathophysiological similarities with infection in humans, yet does not fully mimic natural infection	[125,126]
Pig	Seeding of pathogen through contact lenses on intact corneas	Successful infection exhibits similar pathophysiological profile to infection in humans, including lesion signature	Infection can be induced without corneal debridement; easier contact lens application and manipulation; anatomical and histological similarity of eye to human	Infection is self-limiting with spontaneous remission, unlike in human infections	[67]

[a] Take-home message: an effective treatment should show sufficient killing kinetics against the trophozoic stage of the amoeba during infection of the ocular surface, such as the cornea. In that sense, most mammalian ocular environments might represent an acceptable stratum for an *in vivo* proof-of-concept experiment. However, practical considerations, as well as the degree of pathophysiological similarity to human infection, make pig, followed by rabbit or hamster, the models most preferred by COMP.

Leishmaniasis

Leishmaniasis is caused by a heterogeneous group of protozoan parasites of the genus *Leishmania*, with visceral leishmaniasis [(VL) or kala-azar (VL)[most commonly caused by *Leishmania donovani* and *Leishmania infantum-chagasi* [65,66]. The main route of transmission is via the bite of the phlebotomine sand fly. Occasionally, infection occurs congenitally or through blood transfusion or organ transplantation. *Leishmania* invade and replicate within host macrophages, evading innate and cell-mediated immune responses. Infection generally appears to persist after clinical cure of the primary infection [67].

Leishmaniasis comprises a variety of clinical syndromes, including skin lesions (cutaneous leishmaniasis, CL), recurring and irregular fever, loss of appetite, weakness and fatigue, weight loss, splenomegaly, hepatomegaly and lymphadenopathy, pancytopenia (VL), disfiguring lesions on the soft tissues of the mouth, nose and throat (mucocutaneous leishmaniasis; espundia), and post-kala azar dermal leishmaniasis (PKDL), which appears 6 months to 1 or more years after apparent cure of VL. VL is an opportunistic infection in patients with HIV/AIDS or other causes of cell-mediated immunosuppression and is potentially life threatening without treatment in both immunocompetent and immunocompromised patients.

VL is endemic predominately in developing countries in Latin America, East Africa, and South-East Asia. In Europe, most cases of VL occur in Mediterranean countries, and among immunocompromised patients.

All OMPDs presented to COMP focused on the treatment of VL as a chronically debilitating and life-threatening disease variant. Models for VL are different to those for CL and are summarized in this section. Generally, in the process of assessment of an OMPD application, COMP considers that the selection of a model should be appropriate for the leishmaniasis variant targeted in the development of the medicine. For instance, if CL was proposed as an orphan indication, proof of concept in an appropriate model for this condition would be needed (Table 9).

Given that dogs are a reservoir of *Leishmania*, they represent a naturally occurring animal model of VL (Table 9). However, because of the heterogeneity of naturally occurring *Leishmania* strains, dog breeds, and clinical conditions, this model is expected to be practically more challenging. In addition, as a big animal, dogs can be limiting in terms of experimental numbers in cohorts. Thus, rodents might be more accessible. By contrast, it is more difficult to generate leishmaniasis in rodents and some observations made in these models might not be similar or relevant to humans because of the phylogenetic distance to humans. Hence, the choice of model should be motivated by the aspect of the condition targeted by the medicine, accuracy of the endpoints tested, and ethical considerations. The choice of mouse strain, parasite genotype, and standardized study protocols are important for the successful generation of *in vivo* nonclinical data [71]. For example, outbred mouse strains are generally resistant to *L. donovani* infections [72].

Malaria

Malaria is a serious relapsing infection in humans, endemic to tropical and subtropical regions of the world. It is caused by five recognized species of the related protozoan parasites of the genus *Plasmodium* that are known to affect humans, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium falciparum*, and, less commonly, *Plasmodium knowlesi* [73,74]. The parasite is transmitted to humans by the mosquito vector when it feeds on human blood. The immature form of the parasite, sporozoites, enter the human bloodstream, passing through the bite wound and, once inside the host, the parasite rapidly multiplies by asexual reproduction in the liver. During this latent period, the asexual forms, called merozoites, are formed and emerge from the liver into the peripheral blood, causing the symptomatic disease course. Typically, symptoms occur 10–28 days after infection. The first clinical signs can be any combination of chills, fever, headache, muscle ache, nausea, vomiting, diarrhea, and abdominal cramps. Chills and fever occur in periodic attacks. Severe malaria is more acute, with signs of organ dysfunction and/or high level of parasitemia [74].

All OMPD applications received by COMP aimed to treat severe malaria, where treatment options remain limited and the disease is life threatening. Nonclinical models presented in OMPD applications included rodent and NHP models of severe malaria, most of which are generated with nonspecific-to-human strains of *Plasmodium* (Table 10). Such models were accepted by COMP as supportive of the medical plausibility of the product in treatment of severe malaria. However, data from such models would be considered insufficient to support significant benefit over authorized antimalarial medicines because of difference in the parasites causing human disease. Therefore, all successful applications to data have included also clinical data, which allowed the assessment of the relative efficacy of the proposed medicine in the context of the current standard of care.

The existing models of malaria most often recapitulate many but not all features of human disease. However, they can be used with success for screening candidate drugs, especially if transgenic *Plasmodium* parasites are utilized. This could be useful for, for example, when the medicine is a vaccine or targets the mechanism of *P. falciparum* sequestration (the adherence of infected erythrocytes to the endothelium of blood vessels [75]) (Table 10). It is difficult to reproduce human cerebral malaria in rodent models and, thus, the only rodent model described to recapitulate cerebral malaria, *P. berghei* ANKA in mice, should be explored if the activity of the medicine is meant to target this clinical presentation [76]. Importantly, in rodent models, malaria can clear itself and one has to study the drug effect before the expected natural clearance and with inclusion of an appropriate vehicle control. Therefore, the high-quality reporting of the study protocols would be considered important for COMP assessment. Also, the choice of clinically relevant endpoints should match the symptoms developed by the given model and the endpoints should be of functional relevance to disease in humans (Table 10).

Discussion

Rare infectious diseases might affect limited numbers of European citizens, but they represent a major public health issue for several reasons: (i) some are endemic in certain European regions; (e.g., leishmaniasis and drug-resistant TB); (ii) some have the potential to cause large and lethal epidemics (e.g., EVD infection); (iii) some are agents of bioterrorism (e.g., anthrax or smallpox); and (d) some are neglected diseases affecting mainly the developing world, while rarely seen among returning European travelers (e.g., malaria). Moreover, climate change and improved travel options of limited duration both for human and animal carriers are expected to have an impact on the incidence, prevalence, and distribution of infections acquired through various routes (arthropod vector, rodent, water, food, and air), and many infectious diseases that are currently considered as rare in Europe may re-emerge as impending threats [77].

Neglected tropical diseases affect more than 1 billion people, primarily low-income populations (poor, living in remote, rural areas, urban slums, or conflict zones) and these diseases have a low status in public health priorities in the developing world. There is interest in driving pharmaceutical development to the area of Neglected Tropical Diseases (NTDs) in the European regulatory system, and the EMA, in cooperation with the WHO, has a mechanism to provide scientific opinions on human medicines, including vaccines, that are intended exclusively for markets outside of the EU, under what is usually designated an 'Article 58' procedure [78]. In addition, in this therapeutic area where few incentives exist, the European Orphan Drug regulation could offer research and development incentive for sponsors seeking the development of medicines for neglected communicable diseases (e.g., Ebola or Zika virus). Orphan-designated medicines profit from enhanced development support via the EMA protocol assistance scheme, reductions in regulatory fees, along with other incentives. Examples for such support through the European orphan framework are orphan drugs for the development of medicines for the treatment of Ebola infection and of other life-threatening diseases, such as malaria.

Regulatory support for the development of medicines in rare infectious diseases can be considered useful when recognizing that the development of new drugs and vaccines is challenging and human data on pathophysiology, clinical spectrum, laboratory findings, and therapy are scarce, often derived from case reports and small case series. In addition, for many pathogens, the resultant disease is potentially lethal or permanently disabling for humans and, therefore, research using humans is not feasible. Thus, the development of safe and efficacious vaccines and antimicrobials must rely on the use of appropriate animal models available to researchers. The application of Koch's postulates early in the history

TABLE 9

Models of VL[a]

Animal model	Method of generation	Features of the model	Advantages of model	Disadvantages of model	Refs
Mouse: BALB/c, C57BL/6	Intradermal or intravenous infection with *Leishmania donovani, Leishmania. major or Leishmania infantum* amastigotes	Disseminated granulomas with parasitized macrophages; infection slowly resolves after 4–8 weeks in cure types; low dose infection induces less immune protection, intradermal infection yields higher parasite load	Useful to study immunology, vaccines, chemotherapy, examines activity of drug against liver infection	Does not exhibit the spleen infection, can self-cure in some strains	[127–130]
Mouse immunodeficient SCID or nu/nu	Intravenous infection with *L. donovani* amastigotes	No induction of macrophage activation, because of lack of immune response, atypical granulomas form later	Useful to study leishmaniasis in immunosuppressed cases	Limited applicability in context of clinical immunocompetence	[131,132]
Hamster: Chinese hamster, Syrian golden hamster	Intradermal or intracardial infection with *L. donovani* amastigotes	Inability of infected antigen-presenting cells to stimulate specific T cells, macrophage impairment, progressive disease leading to death	Useful to study pathology and chemotherapy; more synchronous infection in liver and spleen that can develop into a chronic noncure infection more similar to human VL	Lack of available reagents	[133–135]
Rat: African white-tailed rat	Intraperitoneal infection with *L. donovani* or *Leishmania braziliensis*	Long-term disease, good for maintenance of parasites	Excellent host for *in vivo* maintenance and long-term experiments with *L. donovani* and *L. braziliensis*	Not specifically noted in consulted literature	
Rat: Cotton rat (*Sigmodon hispidus*)		Persistent infection, progressive lethal disease	Some of the most susceptible animal hosts for *L. donovani*		[136,137]
Rat: M. natalensis, a multi-mammate rat					[138]
Dog: different breeds	Natural infection with *Leishmania chagasi* or experimental intradermal or intravenous inoculation of *L. donovani* amastigotes	Some infected dogs remain asymptomatic. Lymphoadenopathy, weight loss, anemia, hypergammaglobulinemia, and dermatitis leading to death	Useful to study pathology, vaccines, chemotherapy; dog is not a reservoir of *L. donovani*; reproduces natural infection similar to human disease	Limited for ethical reasons; availability of naturally infected dogs might be limited	[130,139–141]
NHPs: Langurs, vervet monkey, rhesus monkey, mandrills, owl monkey, baboon, marmoset, squirrel, Sykes monkey	Intravenous inoculation of *L. donovani* amastigotes	Owl and squirrel monkey develop acute, fulminant but short-lived infection; Rhesus monkeys develop low burden or inconsistent infections; Langurs develops a progressive acute and fatal disease, similar to human kala-azar	Useful to study pathology; Indian langur presents all clinical immuno-pathological features of human kala-azar. Normally used to study vaccines	Limited use for ethical reasons	[73,142–144]

[a] Take-home message: taken together, COMP would find mouse models of leishmaniasis acceptable because of pathophysiological similarity as well as accessibility. Dog models would be viewed as more accurate representations of human disease, but would not be required on ethical grounds.

of microbiology underlines the significance of animal models in the study of infectious diseases. Nowadays, and after the implementation of the marketing authorization under exceptional circumstances in the EU [79] and the 'Animal Rule' by the FDA [80], animal models are used to provide nonclinical safety and efficacy data for the evaluation of most new antimicrobial agents. This is considered acceptable provided that the applicant describes the relevant principles of medical ethics with precise reference to internationally accepted guidelines on ethics [81].

Medical plausibility at the time of initial orphan designation

According to the European Orphan Regulation, the applicant can apply for an OMPD at any stage of product development as long as the 'intent to treat/prevent or diagnose' can be demonstrated. This intent to treat, otherwise phrased as 'medical plausibility', requires a certain level of evidence, which allows for making an assumption of a disease-relevant activity of the medicine in the condition, as applied for [81]. In the context of infectious diseases, it is expected that nonclinical data need to be generated in appropriate models of the condition (Fig. 1).

In vitro data on the efficacy of a new anti-infective agent might be an alternative to animal models, because they are already usually the first building block for proving activity of a potential new anti-infective. COMP is aware of the development of complex systems for *in vitro* testing of, for example, pathogen clearance/load or mathematical and computer modeling systems. For the acceptability of such new methods, validation with regard to the clinical translatability and clinical relevance is crucial. Generally, *in vitro* data can be used along with animal *in vivo* data for better establishing the efficacy of a new agent, but, currently, *in vitro* data alone would be only exceptionally accepted by COMP (Fig. 1). This could be the case if no relevant *in vivo* model can be generated and there were no medicinal products addressing the disease, or if *in*

TABLE 10

Models of malaria[a]

Animal model	Method of generation	Features of the Model	Advantages of model	Disadvantages of model	Refs
Rat	Intravenous inoculum [*Plasmodium berghei* (Pb)-parasitized erythrocytes] using parasite strain passaged three times through rats	Parasitemia peaking on day 11, 21 days of disease length, high degree of self-clearance, 17% mortality	High infection rate, good for study of infection kinetics and parasitemia inhibition	High rate of self-clearance of parasitemia; surrogate models needed because Pb does not infect humans	[145]
Mouse	Intradermal or intravenous inoculum of parasitized erythrocytes [*PB ANKA* (PbA); but *Plasmodium yoelii, Plasmodium chabaudi, Plasmodium vinckei* also infect mice]	Severe disease caused by PbA, with neurological symptoms or coma, strong immune reaction, acute lung and liver pathology, metabolic acidosis, fatal outcome	Relevant models for studying mechanism of immune response, antimalarial of general mechanism of action not specific to human malaria; PbA model relevant to study cerebral malaria	High rate of self-clearance of parasitemia; surrogate models needed because PbA and others do not infect humans	[77,129]
Rodent	Parasites that express *Plasmodium falciparum* VAR2CSA or CSA binding domains on the surface of infected red blood cells (iRBCs); transgenic *P. berghei* parasites in rodents	Antibodies developed in mice or rats, but screening of antibody affinity done only *ex vivo* so far	Such parasites in combination with a 'humanised placental malaria mouse model' might offer screening system for *in vivo* testing of inhibitors that block *P. falciparum* sequestration	Surrogate models in absence of *in vivo* activity screening	[146,147]
Mouse: NOD/SCID or FRG (humanized)	Engrafted with human erythrocytes or hepatocytes; intraperitoneal inoculation of *P. falciparum* 3D7-infected red blood cells	Depending on model, only some stages of *Plasmodium* life cycle can be reproduced; parasites accumulate in several organs	Good for research of medicines in immunocompromised setting; research of antimalarials targeting erythrocytic stages	Parasitemia not maintained; whole life cycle of parasite not easily reproduced	[148–152]
Gerbil	Infected by PbA via intraperitoneal injection of infected red blood cells	Weight loss, hypothermia, anemia, splenomegaly, hepatomegaly	Similarity with human condition (splenomegaly, hepatomegaly); pathogenesis of severe malaria can be studied	No involvement of nervous system; limited use because of paucity of reagents specific to gerbil	[153]
NHPs: *M. mulatta*, olive baboon, Rhesus monkey, *Saimiri* and *Aotus* monkeys	*P. knowlesi*, *Plasmodium cynomolgi, Plasmodium vivax, Plasmodium ovale* etc.-parasitized erythrocytes	Severe disease, almost always lethal; mimics *P. falciparum* infected red blood cell sequestration and resetting; cerebral infected red blood cell sequestration and neurological signs; full life-cycle of parasite reproduced	Good model of human cerebral malaria; large overlap between antibody isotypes of *Aotus* and human makes this model applicable for vaccine screening and could complement research in other models	Limited use for ethical reasons; limited availability of reagents specific to NHPs	[154,155]

[a] Take-home message: appropriate mouse models exist that can be used to demonstrate medical plausibility in malaria. Careful study design would be required to avoid the pitfalls associated with known limitations of these models.

vitro tests were considered adequate for the intended context of use and support clinical translatability (Fig. 1).

Of 60 applications reviewed for this analysis, 24 contained non-clinical data only, indicating that 40% of applications for rare infectious diseases were for products that had not yet been evaluated in humans. Significant benefit was not required in 38% of the designated conditions. Interestingly, these were mainly viral diseases, suggesting a higher unmet need in these conditions. This review of previous COMP assessments showed that the selection of an appropriate animal model is vital. If there is no possibility to generate clinical data, the need for animal models of higher order (dogs or NHPs) might be exceptionally required, and this is assessed on a case-by-case basis. For example, it might be impossible to test Ebola vaccines and treatments in NHPs or in patients. The selection of the appropriate pathogen (genus, species, and strain) is as crucial as the selection of the animal model. As an example, the Reston strain of Ebola virus that causes disease and death in primates does not cause disease in humans [82] and, therefore, cannot be accepted in an animal model. In general, laboratory-adapted strains of pathogens tend to become attenuated through successive cultures in artificial media, whereas clinical strains better mimic the human condition. The use of human unspecific disease strains (e.g., rodent specific strains of *Plasmodium*) must be justified and contextualized with regards to, for example, the common mechanism of the activity of the medicine. The use of a model of a different disease would be only accepted if the generation of an appropriate model was technically challenging and ethically questionable (e.g., as in the case of smallpox infection).

Disease-relevant endpoints

This review of previous COMP assessments also demonstrates that the time points for therapeutic intervention and the efficacy assessment in animal models should accurately reflect the human disease presentation. In many cases, the animal models might not

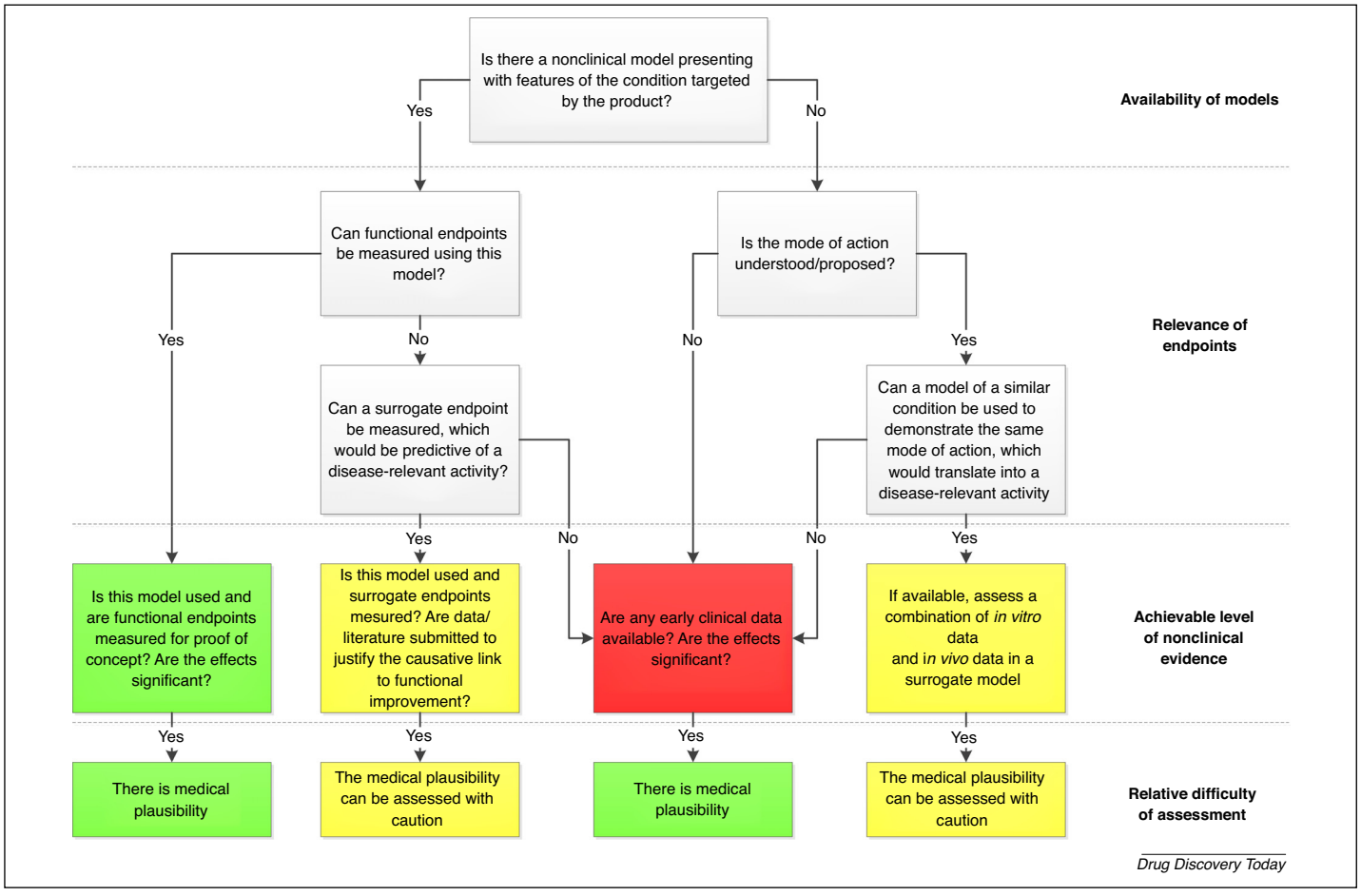

FIGURE 1

Schematic illustrating the process of assessment of medical plausibility by the Committee for Orphan Medicinal Products (COMP). Reproduced from [10].

have symptoms, and the progression of disease or time course of clinical signs might not be the same as in humans. COMP strongly encourages the use of efficacy endpoints that can be linked to the human pathology and symptoms. Often, infectious disease research studies cause considerable animal pain or distress. As an example, some studies require infected animals to exhibit significant clinical disease, similar to that observed in infected humans, before administration of an antimicrobial agent, or in the case of controls, no antimicrobials are administered. Therefore, endpoints should be clearly defined to allow the earliest removal of an animal from an experiment, with the goals of preventing unnecessary animal suffering while achieving the desired scientific results. Animal death should be used as an endpoint only exceptionally, because earlier endpoints should be preferred when applicable. Nevertheless, in several types of infection, it was the survival that was perceived as the most informative functional outcome in existing nonclinical models (Table 1).

Standardization of nonclinical tests

The assessment of nonclinical studies in OMPD applications was sometimes challenging because of the limited information submitted about the nonclinical models used or on the methods used in the experiments. Therefore it is vital that studies are conducted in accordance with state-of-the-art scientific standards and that all relevant information is reported in the submission document, as exemplified by documents such as the ARRIVE guideline [83]. Details such as animal genetics, the species and strain of pathogen, the experimental procedures, supportive care, medications, and the relevance of endpoints to human should be described fully, to allow COMP to assess the application with the scientific rigor applicable to the early stage of development. The application of standardized study protocols and studies designed to be informative for the intended clinical use of the medicine [83] would be in line with the spirit of the 'replace, reduce, refine (3Rs) of animal research' efforts [9,84].

Concluding remarks

COMP required either animal model studies or clinical data for most of the 60 applications for rare infections included in this analysis. Based on COMP experience, the selection of the appropriate animal model for each infection should be guided by multiple factors, such as the characteristics of the animal species and the infecting organism, the similarity of the experimental infection with human infection in terms of disease course and symptoms, and the endpoints that can be used in the animal experiments and their translational value for the human situation (for a graphic summary see also Fig. 1 in [10]). In view of the need to eventually replace animal experiments, COMP would support the development of *in vitro* models, their standardization, and validation. Applicants should

always be aware of the potential limitations of their models and extrapolate cautiously their findings to the human condition. In the experience of COMP, it is vital that the applicants adhere to the animal welfare guidelines and comply with applicable regulations, and are encouraged to fully report all experiment details [83].

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GLOSSARY

Disease-relevant endpoint measurement in a nonclinical study that can be translated into therapeutic activity. This is most often a functional endpoint, but can sometimes be a well-established intermediate measure (e.g., neuronal connectivity, conduction velocity, etc.).
Disease-relevant activity here understood as the pharmacodynamic (PD) activity of the product, which translates into improvement in functional and disease-specific endpoints. This term is used in the context of nonclinical data and is conceptually equal to the term 'efficacy', which is reserved for clinical development.
Medical plausibility a demonstration of the intent to treat the proposed condition using the proposed medicinal product. For the demonstration of medical plausibility, the sponsor has to provide data in patients or in a model of the condition (usually in vivo) that show a disease-relevant PD activity of the medicinal product.
Orphan medicinal product designation (OMPD) a status awarded to a medicinal product in development once eligibility criteria laid down in the orphan legislation are met. In Europe, the eligibility is assessed by COMP and the positive opinion is then considered by the EC for the entry into the European orphan medicinal products register.
Significant benefit a requirement specific to the European orphan legislation, required when other medicines are authorized for the treatment of the same condition. Significant benefit can be understood as a clinically relevant advantage (e.g., improved efficacy) or a major contribution to patient care (e.g., an improvement affecting quality of life).
Surrogate disease model a model of a disease, with only a limited representation of disease features (e.g., similar pathophysiology and only one of the disease features).