Advances in Preventive Medicine

Recent Advances in Prevention and Control of Rabies

Guest Editors: S. N. Madhusudana, Deborah Briggs, and Hervé Bourhy

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Editorial Board

Contents

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Editorial

Recent Advances in Prevention and Control of Rabies

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Rabies is one of the oldest zoonotic disease which continues to pose a significant threat to humans in most parts of the world, particularly in Asia and Africa. As huge animal reservoirs exist in most parts of the world, the threat to humans is likely to continue for many more years. In the past decade, a renewed effort has been made to reduce the burden of rabies focusing particularly on the Asian and African countries. Several national and international organizations including World Health Organization (WHO) and Global Alliance for rabies Control (GARC) are now actively working out various strategies for getting international and national commitment to eliminate human rabies and reduce canine rabies. New developments have taken place in rabies prevention in humans and canine rabies control. Keeping this view, a special issue of Journal of Preventive Medicine was designed to update the readers on important aspects of rabies prevention, epidemiology and control. There are six well written and informative papers in this issue. The first paper by G. Gongal and A. E. Wright deals with the current rabies scenario in the world with special reference to south east Asian countries which contribute nearly 60% of global burden. The paper describes the current trends in human and animal rabies in this region and newly initiated preventive and control efforts to eliminate dog-mediated human rabies in this region. The second paper by B. Dodet et al. gives an insight into the rabies situation in eastern Europe and Middle East. The paper deals with the outcome of a meeting of these countries which have recently joined hands in forming a Middle East and Eastern Europe Rabies Expert Bureau (MEEREB). It highlights the continued presence of canine rabies particularly in Egypt, Turkey, Iran, and Ukraine where several human deaths have occurred. The number of postexposure treatments is also on the increase in these countries Iran alone is administering more than 200,000 PEPs to humans bitten by dogs.

The third paper by J. M. Reynes et al. describes a collaborative study between Pasteur Institute, Madagascar and Pasteur Institute, Paris on the situation of rabies in humans and animals in the island country of Madagascar located in the Indian Ocean in the eastern coast of Africa. The study analyses the rabies situation between 2005 and 2010 and the incidence of canine rabies was 54% and 9 confirmed human deaths occurred. Interestingly, rabies was not reported in bat species though one bat species did had antibodies to Lyssavirus.

Though intradermal rabies vaccination (IDRV) with modern cell culture rabies vaccines is now widely used in many developing countries, the minimum potency requirement for IDRV is not very well documented and some countries have opted for a higher antigenicity (>2.5 IU per intramuscular dose). In an interesting study reported here by D. Brown et al. from the UK, it is clear that 100% seroconversion with antibody titers equal to or greater than 0.5 IU/mL can be achieved if a minimum cumulative antigenic content administered over a period is at least 2 IU. However, the results are based on a very small number

of subjects and a larger study of this kind may help us understand the kinetics of immune response to varying antigenic content and dosage schedule of IDRV.

Another interesting observation with regard to IDRV has been reported by T. Kamoltham et al. from Thailand. Here, they have compared the anamnestic response to booster doses of vaccine in school children who had received 2 (day 0 and 28) or 3 doses (days 0, 7, and 28) of preexposure primary vaccination in the past. They have shown that 2 doses by themselves induced protective levels of antibodies and the anamnestic response to booster doses administered 1, 3, or 5 years later was more or less, the same both in 2-dose and 3-dose primary groups. This aspect needs further investigation, as it has some implication in the cost of preexposure vaccination in children in rabies endemic countries.

In recent years, more and more information has accumulated on the structure and functions of rabies phosphoprotein (P). It has been shown that it is involved in the induction of innate immunity by promoting interferon production. Some groups are targeting P for genetic alteration and creating effective live attenuated vaccines for animal use. In a very interesting study, A. Vos et al. from Germany have created a P gene deletion mutant of SAD B 19 strain of rabies virus that seems to have a potential for use as live attenuated virus for immunization of animals. Such studies are indeed required to produce live vaccines that are not only highly immunogenic but also safe for untargeted animal species, specially humans.

Over all, this special issue on rabies contains timely, informative, and thought-provoking papers that may be of interest not only to basic researchers but also to medical and veterinary professionals engaged in prevention of human rabies and control of canine rabies.

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Review Article

Human Rabies in the WHO Southeast Asia Region: Forward Steps for Elimination

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There are eleven Member States in the WHO southeast Asia region (Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand, Timor-Leste) of which eight are endemic for rabies. More than 1.4 billion people in the Region are at risk of rabies infection, and approximately 45% of worldwide rabies deaths occur in Asia. Dog bites account for 96% of human rabies cases. Progress in preventing human rabies through control of the disease in dogs has been slow due to various factors. Innovative control tools and techniques have been developed and standardized in recent years. The introduction of cost-effective intradermal rabies vaccination regimens in Asian countries has increased the availability and affordability of postexposure prophylaxis. Elimination of rabies is not possible without regional and intersectoral cooperation. Considering the importance of consolidating achievements in rabies control in Member countries, the WHO Regional Office for southeast Asia has developed a regional strategy for elimination of human rabies transmitted by dogs in the Region. They have committed to provide technical leadership, to advocate national health authorities to develop major stakeholder consensus for a comprehensive rabies elimination programme, and to implement national strategies for elimination of human rabies.

1. Introduction

Rabies is an ancient viral zoonotic disease that is invariably fatal in humans and mammals. The disease circulates in two epidemiological cycles: an urban cycle involving maintenance of infection in dog populations and a sylvatic cycle involving wildlife. There is a possibility of spill-over of rabies virus from dogs to wildlife and vice versa.

Dogs are the most important rabies reservoir. Human cases have also been reported due to exposure to rabid cats and wildlife. Mongoose (*Herpestes* spp.), jackals (*Canis aureus*), foxes (*Vulpes bengalensis*) and wolves (*Canis lupus*) have been incriminated as wildlife reservoirs of rabies in Bangladesh, India, and Nepal [1]. Recent studies on the *Nepalese* field rabies virus indicate that it belongs to the Arctic fox genome [2]. The rabies virus isolated from a human rabies case was 100% identical to viruses isolated from two dogs and a mongoose in Nepal [3].

Dog bites are the primary source of human infection in all rabies endemic countries and account for 96% of rabies cases in the southeast Asia (SEA) region [4]. Elimination of human rabies is dependent on elimination of dog rabies.

Countries can be categorized depending on rabies status: high, medium, and low rabies endemic countries and rabies-free countries. Maldives, Timor-Leste, and some islands of India are historically free of rabies. Bangladesh, India, and Myanmar are high rabies endemic countries. Bhutan, Nepal, and Sri Lanka are medium rabies endemic countries. Thailand is moving towards low endemic status, but due to increasing rabies incidence Indonesia is moving from a low endemic to a medium rabies endemic country. Rabies is an emerging disease problem on many islands of Indonesia which were previously considered rabies-free. Some countries have a comprehensive rabies control programme but it is a neglected disease problem in others due to competing public health priorities and the complex nature of rabies control activities.

Prevention of rabies in humans depends on a combination of interventions. These include provision of postexposure prophylaxis (PEP) to exposed patients, preexposure

immunization of people at high risk of exposure, control of infection in animal reservoirs, and control of dog populations [5]. Although rabies is preventable, the high cost of vaccines, compounded by the lack of education and awareness about the disease, limits the use of PEP. Recent studies show that most patients were victims of rabies due to negligence, ignorance, or the inadequate availability of primary health care services [4].

Progress in preventing human rabies through control of the disease in its animal reservoir has been slow. This has been due to technical, intersectoral, organizational, and financial obstacles. In addition, there has been a lack of efficient dog rabies control campaigns including humane canine population management [6]. The success and sustainability of dog immunization coverage depends heavily on appropriate management of the dog population. The efforts towards population management are limited and disjointed in most countries. Lethal methods of dog population control have been used in some countries which have been an expensive option. Attempts to control rabies through dog culling have not been sustainable or socially acceptable due to public, religious, and animal welfare concerns. Furthermore, surgical sterilization of dogs in small numbers and at irregular intervals does not yield any long-term benefits in reduction of the population. There are successful programmes of dog population control in limited urban areas coordinated by leading NGOs. However, they are location specific and have not been replicated at rural levels with community participation.

2. Burden of Disease

Rabies is a disease of public health and economic importance in southeast Asia. The annual expenditure due to rabies has been estimated to be more than US$ 563 million in Asia [7]. This figure is based on the direct and indirect costs of PEP in humans and costs incurred from dog rabies control efforts.

Rabies is a disease of poverty, affecting vulnerable populations and children. According to available data, children in the 5–15 year age-group represent about 40% of people exposed to dog bites in rabies endemic areas [4]. The majority of bites that occur in children go unrecognized and unreported and, consequently, exposed children do not receive the benefit of timely and complete courses of postexposure prophylactic treatment [8]. Additionally, paralytic rabies is often misdiagnosed as acute neurological syndrome. Thus, there is the possibility of a disproportionately high number of young children contracting and dying of undiagnosed rabies.

More than 1.4 billion people are at potential risk of rabies infection in the southeast Asia region. Table 1 shows countrywise estimates of human rabies and dog bite cases.

Each year, 21 000–24 000 people die in the SEA Region due to rabies. This accounts for approximately 45% of worldwide human rabies deaths.

Of the estimated 19 million humans bitten by dogs in the SEA Region, it is estimated that at least 4 million receive one or more doses of rabies vaccine [9]. In the majority of countries, the number of patients receiving PEP has steadily increased over time, particularly in urban areas. This is due to improvements in awareness, availability, affordability, and accessibility of safe and effective rabies vaccines particularly in urban areas. Countries are allocating increasing portions of health budgets to procurement of modern rabies vaccines and immunoglobulin to meet the growing demand for PEP.

3. Feasibility of Elimination of Human Rabies

The necessary tools and methods for prevention and control of human and canine rabies are available. The proof of the feasibility of elimination of dog-mediated rabies has been demonstrated in countries like Singapore and Malaysia. It is thought that strict enforcement and policies of dog registration, vaccination, and dog population management have made rabies control and eradication effective in these countries. Malaysia borders Thailand, and the concept of an immune belt has been developed by dog licensing and mandatory vaccination of dogs as well as systematic destruction of unvaccinated dogs in a buffer zone to prevent entry of rabies from the northern border.

Sri Lanka and Thailand have registered a decline in the number of human rabies deaths through implementation of a mass dog vaccination campaign, improved accessibility to PEP, and effective vaccine delivery systems.

Control of rabies through vaccination in the canine population is fundamental to elimination of human rabies. Rabies elimination programmes focused mainly on mass vaccination of dogs are largely justified by the future savings of human rabies prevention programmes. The Pan American Health Organization initiated a regionally coordinated programme for elimination of human rabies transmitted by dogs in 1983. This was mainly based on mass immunization of dogs and has led to a 90% reduction in and elimination of dog rabies from Chile and major urban centers of other Latin American countries [10]. In Mexico, after five years of a nationwide dog vaccination campaign, the number of human rabies deaths was reduced from 60 per year to less than 20 [11].

Coordinated mass dog vaccination campaigns will improve herd immunity levels and prevent potential human exposure to rabies but strong political commitment and intensive social mobilization is vital. The active role of the veterinary authority at the national level for animal rabies control is crucial and it is their social responsibility to prevent human rabies through well-planned dog rabies control programmes. There are increasing numbers of international partners for dog rabies control and dog population management in southeast Asia which have been encouraged since the introduction of World Rabies Day in 2007.

Innovative tools and techniques have been developed and standardized in recent years which will help to improve dog vaccination coverage, accessibility, and affordability of modern rabies vaccine and dog population management. As an adjunct to parenteral immunization, oral rabies vaccines (ORVs) have been extensively tested for efficacy and safety in owned and ownerless dogs. ORV delivery strategies for dogs which cannot be reached by parenteral vaccination have

Table 1: Distribution per year of human rabies and dog bite cases in countries of the southeast Asia region.

Country	Estimated no. of dog bites	Estimated no. of human rabies cases	Estimated no. of human cases per million population	Source of information
Bangladesh	300,000	2,000–2,500	13	Ministry of Health and Family Welfare, Bangladesh
Bhutan	5000	<10	3	Ministry of Health, Bhutan
DPR Korea	Not available	Not available	Not available	N/A
India	17,400,000	18,000-20,000	18	Assoc. for Prevention and Control of Rabies in India (APCRI)
Indonesia	100,000	150–300	1.3	Ministry of Health, Indonesia
Maldives	0	0	0	N/A
Myanmar	600,000	1000	22	Ministry of Health, Myanmar
Nepal	100,000	<100	4	Ministry of Health and Population, Nepal
Sri Lanka	250,000	<60	3	Public Veterinary Services, Sri Lanka
Thailand	400,000	<25	0	Ministry of Public Health, Thailand
Timor Leste	1,000	0	0	Ministry of Health, Timor-Leste
SE ASIA TOTAL	19,156,000	21,345–23,995		

been designed and tested in parts of Asia [12]. However, the cost of oral rabies vaccine for dog immunization is a limiting factor. The use of immunocontraception may be considered, in conjunction with oral and parenteral rabies vaccination, as a complementary tool to reduce the density of dog populations and rabies incidence [13].

4. Cost Effectiveness of Intradermal versus Intramuscular Rabies PEP and RIG

India is the only country in the Region producing various types of quality rabies tissue-culture vaccines (TCVs). It is capable of producing 15 million doses of rabies vaccine annually (Personal communication, Dr. RL Ichhpujani), which is sufficient for whole region. Use of RIG in category three bites is limited due to the high cost of HRIG administration. Purified ERIG is now produced in sufficient quantities in India and Thailand and is safe and affordable to use. The availability of highly effective human rabies vaccines and ERIG within the region is important to prevent possible human deaths due to exposure to rabid animals.

Cost-effective rabies vaccination schedules were introduced in early 1990s. The WHO Expert Committee in 1991 recommended intradermal application of modern rabies vaccines for PEP [14]. Multisite intradermal administration of rabies vaccines reduces the costs of PEP by 60% [15]. The original Thai Red Cross Regimen can be replaced if two doses of vaccine are given on day 0,3,7, and 28 ("2-2-2-0-2" regimen) as per the recommendation of the eighth WHO Expert Consultation [16]. The updated Thai Red Cross Regimen considerably improves compliance rates as patients receive the full course within one month. A research study is ongoing for the administration of a complete PEP intradermal schedule within the period of one week.

Intradermal rabies vaccination (IDRV) was introduced and widely used in Thailand in the past and has been adapted and promoted in Bangladesh, India, Sri Lanka and other Asian countries. Today, over 95% of patients are provided PEP with an IDRV regimen in Sri Lanka (personal communication, Dr. Omala Wimalaratna). WHO has been providing technical support to other rabies endemic countries to introduce the IDRV regimen.

The cost of PEP can be reduced dramatically if the intramuscular (IM) regimen is replaced in a progressive manner with the IDRV regimen. The conservative estimate of costing for human rabies prophylaxis, including vaccine and serum application for a period of five years in southeast Asia region, is presented in Tables 2 and 3. Vaccine wastage is expected with IDRV due to the short shelf life of the vaccine after reconstitution and fewer patients at the time of vaccination. While calculating the cost estimates for IDRV, it was estimated that 25% vaccine will be wasted.

Table 2 gives cost estimates for the use of IM and ID PEP regimes. If only the intramuscular (IM) PEP regime is used over a period of 5 years, the cost estimation is US $705 million. In contrast, if only the intradermal (ID) PEP protocol is practiced the cost estimation will be almost two thirds less at US $226.5 million. This demonstrates the cost-effectiveness of ID versus IM PEP protocols. Figure 1 illustrates the cost estimate effects of varying the percentage proportion of ID and IM rabies PEP.

Experience has shown that 25% victims have third category bites that require rabies serum application. Equine

Table 2: Cost estimation of intramuscular (IM) versus intradermal (ID) rabies postexposure prophylaxis (PEP) in the southeast Asia region over a five year period.

Activity	100% IM PEP	100% ID PEP
Estimated number of patients taking PEP (millions)	4	4
Volume of TCV required per person for PEP course (milliliters)	5	1
Total volume of TCV required to achieve 100% coverage (+25% wastage for ID) (millions of mLs)	20	5
Cost per millilitre of TCV, incl. syringe + diluent (US$)	7	9
Cost of TCV vaccine per year (million US$)	140	45
Cost of vaccine for five years (million US$)	700	225
Cost of transportation, storage, etc. (million US$)	5	1.5
Total cost for five years (million US$)	705	226.5

(TCV: tissue culture vaccine. Vaccine costs calculated at current market rates. Transportation and storage costs estimated as 25% of vaccine volume).

Table 3: Cost comparison of human rabies immunoglobulin (HRIG) versus equine rabies immunoglobulin (ERIG) for postexposure prophylaxis (PEP) in southeast Asia over a five-year period.

Activity	HRIG	ERIG
Estimated number of patients taking PEP (millions)	4	4
No. of dog bite cases requiring RIG application (millions)	1	1
Quantity of RIG required per patient (millilitres)	5	5
Quantity of RIG required per year (millions of mLs)	5	5
Cost of human RIG per mL (million US$)	60	3
Cost of human RIG for year (million US$)	300	15
Cost of transportation, storage etc. (million US$)	2	1.25
Total cost of RIG for five years (million US$)	1510	81.25

(RIG costs calculated at current market rates. Transportation and storage costs were estimated at 25% of RIG volume).

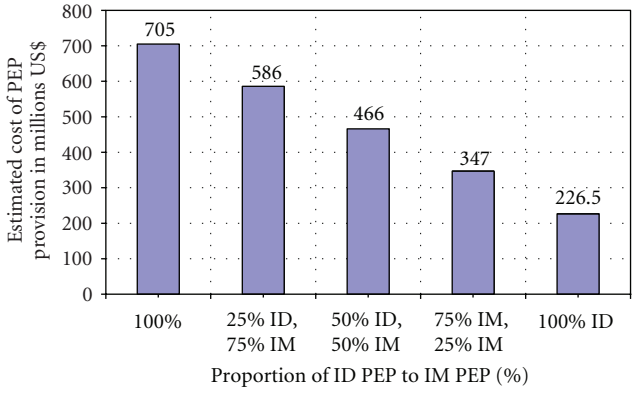

Figure 1: Estimated costs of providing varying proportions of intradermal (ID) versus intramuscular (IM) rabies postexposure prophylaxis (PEP) to 4 million dog bite patients.

rabies immunoglobulin (ERIG) is over 18 times cheaper than human immunoglobulin (HRIG) as shown in Table 3. Using only HRIG in third-degree bite cases over a five-year period will cost approximately US $1.51 billion, whereas using ERIG over the same period will cost approximately US $57 million.

5. Regional Multisectoral Collaboration

Except for island countries, elimination of rabies is not possible without regional cooperation. No single country can maintain rabies-free status unless it is brought under control in neighbouring countries. Regionally coordinated efforts are necessary for elimination of human rabies with consideration of country-specific needs and socio-cultural acceptability. WHO launched a regional rabies control project in the 1980s in Asia and many countries developed and strengthened national capacity for rabies surveillance, diagnosis, vaccine production, and dog population management. This encouraged coordination and cooperation between human and animal health sectors for rabies prevention and control at the country level.

Following the first WHO recommendation in 1984 to replace nerve-tissue vaccines (NTVs), many developing countries of southeast Asia have discontinued the production and use of NTVs for human use [17]. In 2004, the WHO Expert Consultation issued a definitive statement to the effect that NTVs should be discontinued [16]. As a continuous effort of WHO and professional organizations, all countries except Bangladesh and Myanmar have discontinued production and use of the Semple type NTV.

Considering the importance of consolidating achievements in rabies control in Member countries, WHO SEARO came up with the regional strategy for human rabies elimination from southeast Asia in 1998. WHO SEARO organized an intercountry meeting in Colombo, Sri Lanka in 2005 to review the rabies situation in SEA Region and to formulate mechanisms for implementation of the strategy for elimination of rabies [18].

It is important that Member States of the Association of southeast Asian Nations (ASEAN) and the South Asian Association for Regional Cooperation (SAARC) have identified rabies as a priority public health problem. There is also a growing concern and commitment for elimination of human rabies by a number of national governments in the SE Asia Region. There was a SAARC Rabies Meeting in Colombo in 2003 which was attended by government officials of Member countries. The meeting recommended the development of a SAARC strategy for rabies elimination [19]. The ASEAN countries adopted the resolution to prevent and control rabies, with the goal of rabies elimination by year 2020 [20]. The Rabies in Asia Foundation conference held in Hanoi in September 2009 passed resolutions to take several steps to reach the goal of human and dog rabies elimination by 2020. The WHO has been requested to reinforce capabilities to meet the demands from Member States for technical assistance, technology transfer, and the launching of regional initiatives for dog rabies control and elimination in Asia in close collaboration with ASEAN and SAARC [21].

The WHO Strategic Framework for the Elimination of Human Rabies Transmitted by Dogs in the South East Asia Region is due for publication in 2011. The document will provide technical guidance for the regional strategy as well as national strategies and act as an advocacy tool to develop consensus among major stakeholders for a comprehensive rabies elimination programme.

6. Conclusion

The elimination of human rabies transmitted by dogs is an achievable goal. The initiative has been taken to develop a unique strategic framework for elimination of human rabies transmitted by dogs in consideration of the epidemiological situation, technical feasibility, and the sociocultural context. The cost benefits of using intradermal rabies vaccines and equine immunoglobulin for postexposure prophylaxis has been demonstrated. These techniques must be adopted. The WHO Strategic Framework will be a vital guide for the collaborative, intersectoral approach that is necessary for rabies control. With the adoption of the strategic elements of this document the huge public health and economic burden of human rabies can by eliminated in southeast Asia.

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Review Article

Report of the First Meeting of the Middle East and Eastern Europe Rabies Expert Bureau, Istanbul, Turkey (June 8-9, 2010)

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Rabies is a threat in all parts of the world where animal reservoirs persists, including Eastern Europe and the Middle East. Rabies experts from seven Middle East and Eastern European countries (Croatia, Egypt, Georgia, Iran, Serbia, Turkey, and Ukraine) met for two days in Istanbul, Turkey (June 8-9, 2010), to exchange information on the epidemiological situation concerning human and animal rabies in their respective countries and to discuss strategies for rabies elimination and control. They decided to establish a regional network, the Middle East and Eastern Europe Rabies Expert Bureau (MEEREB), a regional network of experts, to increase collaboration in rabies prevention and control at the local, regional, and global levels.

1. Introduction

Rabies is a major public health problem causing approximately 55,000 human deaths every year, mainly in Asia and Africa [1]. It is also a threat in other parts of the world where animal reservoirs persist, as is the case in Middle East countries. Rabies continues to be a significant but underestimated public health concern in the region and the situation in some countries has worsened, given the deteriorating environment [2, 3]. While rabies rarely occurs in humans in most of Eastern Europe, thanks to post-exposure prophylaxis (PEP), it remains present in animal reservoirs, particularly in countries situated at the crossroad of Asia, Africa, and Western Europe [4, 5].

A group of experts in infectious diseases, rabies, and vaccinology from various institutions in seven Middle East and Eastern European countries met for two days in Istanbul. They were invited through the Ministries of Health/national CDCs, or directly through personal contacts. The number of participants/countries was voluntarily limited as the sole aim of this initial meeting was to evaluate the feasibility/interest of establishing a regional network. During this meeting,

participants exchanged information on the epidemiological situation of rabies in their respective countries and discussed specific issues and strategies for rabies elimination and control in their region. They focused on human rabies, but the animal rabies situation was also discussed, since prevention of human rabies requires the control of rabies reservoirs in animals.

Participants agreed to establish an informal regional network—the Middle East and Eastern Europe Rabies Expert Bureau (MEEREB)—to complement the Asian Rabies Expert Bureau (AREB), established in 2004 [6–8] and AfroREB, a network of experts from French-speaking Africa, established in 2008 [9, 10].

2. The Rabies Situation

The rabies situation presented during the meeting is summarized in Table 1. All countries represented at MEEREB reported animal rabies, and in some countries where canine rabies is prevalent, the disease still occurs in humans. Cats also play a role as a vector especially in Ukraine, where, according to the data presented, 12 out of 29 (41.4%) human rabies cases in the last 10 years were transmitted by cats, and in Turkey where the recent reappearance of the disease in this species is a concern.

In Croatia and Serbia, no human rabies case has been reported for over 30 years. The last case in Croatia was reported in 1964, and the last case in Serbia was in 1980: rabid dogs caused both. Since then, there have been two-imported cases of human rabies in Croatia. The absence of cases in Serbia and Croatia can be explained by the fact that rabies is present in wildlife only, vaccination of pet dogs is mandatory, and PEP of animal bite victims is accessible free of charge. However, rabies is still enzootic in the red fox in these two countries, and sporadic cases spill over to other wild animal species and domestic animals. In each country, >1,500 animal-bite victims receive post-exposure prophylaxis (PEP) annually. Human rabies immunoglobulins (HRIG) are locally produced by the Zagreb Institute of Immunology and by the Belgrade Blood Transfusion Institute of Serbia.

The specific rabies situation in *Turkey* was described; dog-mediated urban rabies predominates**,** with foci in the Istanbul region. However, fox rabies has been increasing since 1999, especially in the western Aegean region where the numbers of rabid dogs and foxes are approximately equal, a situation unique to Turkey. Occasional rabies cases are observed in the jackal, particularly around Istanbul. These data have also been published recently [11]. One to two human cases are reported annually.

*In Iran***,** rabies is the most important zoonotic disease and has spread across the country including the central desert areas; the most affected provinces are located in the north-east, east, and south. The country is spending an increasing portion of its health budget on procurement of cell culture rabies vaccines and immunoglobulins to meet the increasing demand for rabies PEP. The number of people receiving PEP in the 300 bite management centres across the country has more than doubled between 1997 and 2009, while the rabies mortality rate has decreased from 0.9 per million people in the 1980s [12] to 0.02-0.03 per million people in recent years.

In *Egypt*, animal rabies is present both in urban areas and rural settlements. Stray dogs are the main transmitters to other animals and humans. The situation has been stable for the last 10 years with an annual number of 80 human rabies cases. Among the countries reporting data at the meeting, it had the second highest human rabies incidence, following *Georgia* where the number of reported admissions for PEP following exposure to potentially rabid animals has been increasing steadily (from ~10,000 in 2000 up to 28,055 PEP in 2008—with a peak of ~48,000 in 2006).

Several of the countries represented receive support from the European Union (EU) for oral vaccination of foxes (Croatia, Serbia, and Turkey) and dogs (Turkey) [11]. Western Europe eliminated animal rabies through compulsory vaccination of dogs and oral vaccination of wildlife [13], but the persistence of rabies in animals along borders is a permanent threat. This is illustrated by the reemergence in 2008 of animal rabies in Italy in an area bordering Slovenia, and it spread through the north-western provinces [14, 15].

3. Rabies Prophylaxis

According to WHO, immediate post-exposure vaccination is recommended for category II exposure (nibbling of uncovered skin, minor scratches, or abrasions without bleeding;) and immediate vaccination and administration of rabies immunoglobulin are recommended for category III (single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks, licks on broken skin, exposures to bats). For categories II and III, thorough washing and flushing (for 15-minutes, if possible) with soap or detergent and copious amounts of water of all bite wounds and scratches should be done immediately, or as early as possible following the bite [16]. Intramuscular vaccination consists of either a 5-dose (1 dose on each of days 0, 3, 7, 14, and 28) or a 4-dose schedule (2 doses on day 0—1 in each of the 2 deltoid or thigh sites—followed by 1 dose on each of days 7 and 21). An intradermal regimen (injection of 0.1 ml at 2 sites—deltoid and thigh—on days 0, 3, 7, and 28) may be used for people with category II and III exposures in countries where the intradermal route has been endorsed by national health authorities [16].

PEP is free of charge for the patient in all countries participating in MEEREB, except Egypt, where rabies immunoglobulin (RIG) is rarely administered, even to patients with category III exposure. In Georgia, PEP is free of charge for children under 18 years of age and adults without social coverage. Modern cell culture rabies vaccines are used intramuscularly (IM) for pre- and post-exposure prophylaxis. Most countries use 5-dose IM regimen (at days 0, 3, 7, 14, and 28). In three countries (Egypt, Georgia, and Ukraine), a 6-dose IM vaccination schedule (with an additional dose at day 90) is applied in compliance with the labeling of some regionally produced rabies vaccines.

Table 1: Rabies epidemiology and management in the 7 countries represented at MEEREB.

	Croatia	Egypt	Georgia	Iran	Serbia	Turkey	Ukraine
Main reservoir(s)	Fox	Dog	Dog, jackal, wolf	Dog, wolf, fox, jackal	Fox	Dog, fox, jackal	Dog, fox
Main vector(s)	Dog, cat	Dog	Dog, cat	Dog	Dog, cat	Dog	Cat, dog
Human population (estimates)*	~4,500,000	~80,500,000	~4,600,000	~76,900,000	~7,300,000	~77,800,000	~45,500,000
Number of human rabies cases reported between 2000–2009 [1999–2009 for Turkey]	0	880	90	62	0	21	29
Human rabies cases reported in 2009**	0	80	6	2	0	2	6
Estimated human rabies incidence per million (2009)**	—	0.99	1.30	0.02	—	0.025	0.13
Number of PEP (2009)	1,750	~200,000	28,055	130,531	1,609	178,250	21,000
PEP incidence per million people (2009)**	388	~2,485	6,100	1,700	220	2,290	461
PEP vaccination regimen	4 doses IM (Zagreb 2-1-1) Rarely: 5 doses IM (Essen)	6 doses IM *at days 0, 3, 7, 14, 28, 90*	6 doses IM *at days 0, 3, 7, 14, 28, 90*	5 doses IM *at days 0, 3, 7, 14, 28*	5 doses IM *at days 0, 3, 7, 14, 28*	5 doses IM *at days 0, 3, 7, 14, and 21 or 28*	6 doses IM *at days 0, 3, 7, 14, 28, 90*
Type of rabies Immunoglobulin used	HRIG (locally produced)	HRIG (private sector)	ERIG (public sector) HRIG (private sector)	HRIG (public sector)	HRIG (locally produced)	ERIG (public sector) HRIG (private sector)	ERIG (locally produced)
Number of PrEP in 2009	200 (population at risk)	N.a.***	Vets (on a voluntary basis)	N.a. (population at risk)	51 (population at risk)	991 (population at risk)	N.a. (population at risk)

Figures reported by MEEREB participants.
*July 2010 estimates—The World Fact Book—https://www.cia.gov/library/publications/the-world-factbook/.
**Calculated from data reported by participants for the population mentioned.
***N.a.: data not available.

Human rabies immunoglobulin (HRIG) is used in the public sector in Serbia, Croatia, and Iran. In the other countries, it is available in the private sector only, while equine rabies immunoglobulin (ERIG) is used in the public sector and produced in Ukraine for local use.

Pre-exposure prophylaxis (PrEP) is administered free of charge to people at high risk of exposure in most MEEREB countries, as recommended by WHO [16]. The exceptions are Georgia and Egypt, where it is provided on a voluntary basis. Those vaccinated pay for the treatment.

4. Discussion

This meeting was a first step in establishing a rabies network in the Middle East and Eastern Europe. During this initial meeting, participants noted the common issues and differences in their respective rabies situation and discussed how they could benefit from sharing their experiences to establish a regular, supranational collaboration to fight rabies.

It was noted that data on human rabies cases are available at the national level, but that they need to be consolidated and made available at the international level. For instance, 2 and 6 human cases were reported to have occurred in 2009 in Turkey and Ukraine, respectively (Table 1), while none was reported to the Rabies Bulletin Europe [17].

Human cases still occur in countries where rabies is present in dogs, in spite of vaccine and RIG availability. Strengthening dog vaccination and dog population control is key to rabies control. It was reported at the meeting that among the 1-1.2 million privately-owned dogs in Iran, approximately 400,000 are vaccinated, while there is no vaccination programme for unowned and stray dogs, the estimated number of which is about 1 million [12]. This results in an estimated 15–20% vaccination coverage for the dog population, which is insufficient, since vaccinating at least 70% of the whole dog population is necessary for dog rabies control [18]. The situation is even less clear for cats.

Therefore, the Blueprint for Rabies Prevention and Control that was previewed during the MEEREB workshop was

enthusiastically welcomed by the participants as it helps and guides those implementing rabies control programmes for dog-rabies elimination with a detailed step-by-step action plan. This paper has been prepared by a global group of rabies experts (Partners for Rabies Prevention) under the leadership of the Global Alliance for Rabies Control (GARC) and is available online and can be downloaded free of charge [19].

Furthermore, MEEREB participants noted the need to increase rabies awareness in their region, and agreed to participate in the World Rabies Day. They also noted the need for timely information for people travelling to rabies enzootic areas (i.e., travellers to their regions and/or people from their regions to other enzootic parts of the world). Recent studies showed that most animal-associated injuries requiring PEP in French travellers occur during visits to Thailand and Turkey—countries for which travellers do not usually seek advice since they are not associated with more conventional travel-associated diseases like malaria or yellow fever [20]. The majority of travellers bitten by animals do not receive adequate PEP, or else they experience a substantial delay before receiving it [20–23].

The participants agreed to meet regularly and to invite experts from other countries of the region to join the MEEREB network, and participate in the next MEEREB workshop.

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Research Article

Laboratory Surveillance of Rabies in Humans, Domestic Animals, and Bats in Madagascar from 2005 to 2010

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Background. Rabies virus (RABV) has circulated in Madagascar at least since the 19th century. *Objectives*. To assess the circulation of lyssavirus in the island from 2005 to 2010. *Materials and Methods*. Animal (including bats) and human samples were tested for RABV and other lyssavirus using antigen, ribonucleic acid (RNA), and antibodies detection and virus isolation. *Results*. Half of the 437 domestic or tame wild terrestrial mammal brains tested were found RABV antigen positive, including 54% of the 341 dogs tested. This percentage ranged from 26% to 75% across the period. Nine of the 10 suspected human cases tested were laboratory confirmed. RABV circulation was confirmed in 34 of the 38 districts sampled. No lyssavirus RNA was detected in 1983 bats specimens. Nevertheless, antibodies against Lagos bat virus were detected in the sera of 12 among 50 *Eidolon dupreanum* specimens sampled. *Conclusion*. More than a century after the introduction of the vaccine, rabies still remains endemic in Madagascar.

1. Introduction

Rabies is a zoonotic disease caused by 11 viral species belonging to the genus *Lyssavirus* (Rhabdoviridae family), including the rabies virus (RABV), the most common [1–3]. These viruses are responsible for a meningoencephalomyelitis in mammals. Transmission of the viruses to a healthy mammal occurs mainly through bite or scratch by an infected mammal (the saliva is the infectious material). Bats are considered as the natural hosts of 10 of these viral species. However, dogs are the main source of infection in humans. It is estimated that 55,000 deaths per year worldwide are due to rabies infection with about 56% of which occur in Asia and 44% in Africa. In Africa and Asia, these deaths are responsible for 1.74 million disability-adjusted life years (DALYs) lost each year [4]. There is no effective treatment when the disease is declared. However, there is an effective treatment against RABV and closed related lyssaviruses when applied as soon as possible after exposure. It prevents the onset of symptom and death and consists of local treatment of the wound, administration of rabies immunoglobulin (if indicated), and vaccinations against rabies [5].

Lyssaviruses are present in all continents with the exception of Antarctica. RABV is the most widespread, widely distributed across the globe, with only a few countries (mainly islands and peninsulas) being free of the disease. Madagascar, an island in the south-western part of the Indian Ocean, does not belong to these exceptions (http://www.who.int/rabies/rabies_maps/en/index.html). Rabies virus has circulated in Madagascar at least since the 19th century. The son of one administrator of the former French Colony was reported dead of rabies in 1896, and his death was one of the reasons of the establishment of the Institut Pasteur in Madagascar in 1898. The first rabies postexposure treatment using rabies vaccine was implemented in 1902. Since that period, several reports have described the rabies situation in the island [6–9]. The last one, covering the 1982 through 1991 period, indicated that the rabies was raging over the 5 provinces of the island and that dogs were essentially the vector of the virus [9]. We report here the

result of the last 6 years of the laboratory surveillance (2005–2010) carried out exclusively by the national authorized laboratory for rabies diagnostic (NLR) at the Institut Pasteur from Madagascar.

2. Materials and Methods

2.1. Samples. Animal samples tested routinely for rabies consisted of brain, head, or corpse of terrestrial nonflying mammals sent by veterinarians, animal health officers and technicians, animal owners, or persons (or relatives) exposed to these animals. Human samples consisted of postmortem brain biopsies or postmortem skin biopsies taken from the nape of the neck, sent generally at +4°C by hospital staff. Upon reception at the NLR, brain biopsies were kept at +4°C and processed within 48 h. Skin biopsies were kept at −80°C till processing.

Furthermore, samples collected from bats were also tested. They were obtained during a survey looking for virus associated to bats. Samples consisted of sera, blood clots, and pharyngeal swabs kept in viral transport medium (VTM). They were sent within 12 hours to the laboratory and then stored at −80°C at their arrival. When the field was far from the laboratory, they were stored in liquid nitrogen and then transported to the laboratory. When tested, each clot was grinded at a 1 : 10 dilution in cell culture medium (DMEM) containing 30% foetal calf serum and centrifuged at 3,000 rpm for 10 min at +4°C. Then pools of up to 10 supernatants or 10 pharyngeal swabs VTM were constituted before testing.

2.2. RABV Antigen Detection. Rabies nucleocapsid detection was performed by fluorescent antibody test (FAT) using rabbit IgG against RABV nucleocapsid (Bio-Rad, Marnes-la-Coquette, France) and performed on the brain postmortem biopsy as the standard [10].

2.3. RABV RNA Detection. RNA was extracted from skin biopsies according to the procedure described by Dacheux and colleagues [11]. RNA was extracted also from pools of bats blood clots supernatants or bats pharyngeal swabs VTM using TRIzol LS (Invitrogen, Carlsbad, Calif, USA) and from brain biopsies using TRIzol (Invitrogen, Carlsbad, Calif, USA), as recommended by the manufacturer.

Lyssavirus RNA detection was performed using a reverse transcription and a heminested PCR targeting a conserved region of the polymerase genes of lyssaviruses [11].

2.4. RABV Isolation. Virus isolation was performed to confirm the negative result of the rabies virus antigen detection in animal samples tested routinely for rabies. From 2005 through 2007, virus isolation was performed in newborn mice [10], then isolation was performed in cell cultures (Murina neuroblastoma cell line) [12].

Virus isolation in new-born mice was also used for the samples collected from bats.

2.5. Detection of Antibodies against Lyssaviruses. Antibodies against RABV, Lagos Bat Virus (LBV), European Bat Lyssavirus type 1 (EBLV-1), EBLV-2, Mokola virus (MOKV), and Australian Bat Lyssavirus (ABLV) were detected in bat sera using lyssavirus rapid fluorescent focus inhibition test [13].

3. Results

3.1. Rabies Virus Detection in Human and Domestic or Tame Wild Animal Samples. During the 6-year period, the NLR received 461 specimens, 450 from animals and 11 from humans. Most of the 450 animal samples were from domestic carnivorous (n = 409, 90.9%), including dogs (n = 353, 78.4%) and cats (n = 56, 12.4%). We noticed that lemurs, an endemic primate from Madagascar, counted for 2% of the animal samples. All lemurs sampled were reared as pets. Brain was available for all animals. Eleven human suspected rabies cases were also laboratory investigated. Human samples consisted of skin biopsy for 6 cases, brain for 4 cases, and cerebrospinal fluid for one case. Fourteen samples were inadequate and could not be tested, mostly because of inadequate storage (Table 1).

Half of the 437 animal specimens tested (all brains) were found positive using FAT. All the samples from lemurs were tested negative. Cattle and pigs, not frequently sampled, were often found positive. More than half of the dogs tested were found infected (Table 1). This percentage varied across the period from 26% (12/47) to 75% (58/77) (Figure 1). When comparing some characteristics of confirmed rabid dogs and RABV noninfected dogs sampled from 2006 through 2010, the positive predictive value was highest for dogs suspected of rabies-clinical disease or unusual spontaneous attack 60.6% (95% CI 53.6%–67.7%), for dogs responsible for bite 50.9% (95% CI 44.3%–57.5%), or for dogs less than 4 years old 57.3% (95% CI 48.9%–65.8%) (Table 2). Nine of the 10 human cases samples tested were found positive (Table 1). The sample tested negative was one skin biopsy.

During the 6-year period, the 447 samples tested were received from 38 of the 111 administrative districts of Madagascar. Most of these samples (365; 82%) were received from Antananarivo province. Rabies circulation was confirmed in 34 of the 38 districts (Figure 2). The virus was present in the capital city of Antananarivo (59 infected animals among 155 tested). Rabies circulation was not detected in 4 of the 38 districts sampled. However, very few samples were received from them (6 samples from one district and 1 sample each from the 3 others).

3.2. Lyssavirus and Antibodies against Lyssavirus Detection in Wild Animal Samples. Brain samples from only two wild terrestrial nonflying mammals were received: one fossa (*Cryptoprocta ferox*), the largest mammalian carnivore of Madagascar, and one roof rat (*Rattus rattus*). They tested negative.

A large collection of samples obtained from insectivorous and frugivorous bats were also tested (Table 3). They were collected during (i) a transversal survey looking for henipavirus carried out in 2004 and 2005 in Madagascar [14] and (ii) a longitudinal survey carried out from 2005 to

Table 1: Rabies laboratory diagnostic in human, domestic and tame wild animals, Madagascar, 2005–2010.

Species	Samples		
	Received	inadequate	Tested positive (%)
Human	11	1	9 (90)
Dog	353	12	185 (54)
Cat	56	1	13 (24)
Cattle	26	0	21 (81)
Pig	3	0	2 (67)
Rabbit	2	0	0
Lemur	10	0	0
Total	461	14	229 (51)

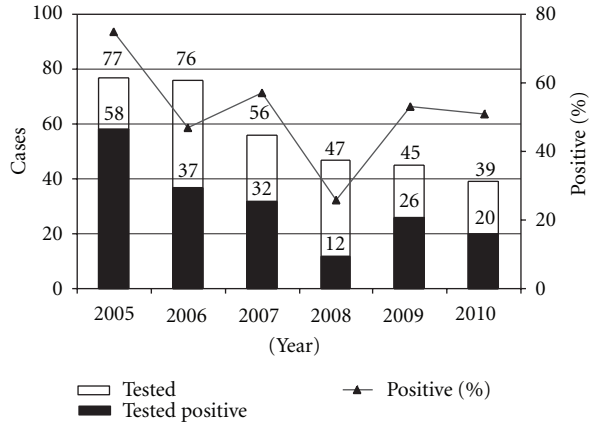

Figure 1: Rabies laboratory diagnostic in dogs, Madagascar, 2005–2010.

2009 in Angavobe and Angavokely caves that host Malagasy straw-colored fruit bat (*Eidolon dupreanum*) (Figure 2). No lyssavirus RNAs were detected in these blood samples and oral swabs. No lyssavirus isolates were obtained from all these samples in new-born mice.

Sera from 28 Malagasy flying foxes (*Pteropus rufus*) and from 50 Malagasy straw-colored fruit bats *(Eidolon dupreanum)* were tested for antibodies against lyssaviruses. Antibodies against EBLV-1 and LBV were detected in five and one Malagasy flying fox, respectively. Antibodies against LBV were detected in 12 Malagasy straw-colored fruit bats (24%), titers ranging from 35.2 to 65. No antibodies were detected against MOKV, EBLV-2, and ABLV.

4. Discussion

Despite the introduction a century ago of the rabies vaccine in Madagascar, the recurrent positive laboratory diagnostic of rabies in dogs suggests that this zoonotic disease remains endemic in the island (Figure 1). The percentage of dogs detected infected by RABV along the 2005–2010 period (54%; 185/341) was in the same range of the one observed during the 1959–1991 period (57%; 1416/2475) [9]. Dogs remain probably the principal vectors of RABV in the island. RABV strains associated to dogs in Madagascar were shown to belong to the cosmopolitan lineage [15, 16]. There was an evidence of RABV circulation in Antananarivo, the capital city. Antananarivo had, in 2007-2008, a density of dogs higher than many other urban areas in Africa, and the dog population was unrestricted and inadequately vaccinated against rabies, this characteristic favouring probably the dissemination of the virus [17]. This situation is probably not limited to the capital city in Madagascar and may explain the rabies endemic situation in the island.

Several endemic or (few) introduced carnivorous mammals (Families Viverridae and Herpestidae) are present in Madagascar [18]. So far, very few suspected animals from these species have been tested. One rabid confirmed human case was bitten by a fossa (*Cryptoprocta ferox*) in Ihosy district, in 2007, and the strain obtained from this case was confirmed as a lyssavirus of the species RABV, phylogenetically closely related to those circulating in Malagasy dogs (data not shown). Consequently, the question of a possible vector in the wild terrestrial carnivorous mammals remains unanswered. This question is of importance considering a rabies control programme targeting the eradication of the rabies in the island.

Our extensive survey in bats failed to detect any lyssavirus associated to these mammals. The molecular technique we used to detect lyssaviruses was demonstrated to be sensitive, reproducible, and repeatable [11]. Furthermore, virus isolation on new-born mice was considered sensitive as we isolated several viruses from the bats specimens, like Ife virus from the Malagasy straw-colored fruit bat (*Eidolon dupreanum*) and Dakar bat virus from the Peters's wrinkle-lipped bat (*Mormopterus jugularis*) (unpublished data). Low prevalence of active infection (detection of virus) has been observed in North American and European bats colonies (0.1 to 2.9%), especially in clinically normal bats [19]. Because we sampled clinically normal bats and because our sampling size per site and per species was for the most about 100 animals (except for the site of the followup where we sampled about 750 animals), our negative results in detecting a lyssavirus are consequently not so surprising. Lyssavirus detection was also negative in brains sampled in 1987 and 1988 in Madagascar, from 59 little free-tailed bats (*Chaerephon pumilus*) [20]. Interestingly, we got serological evidence that lyssaviruses have circulated among Malagasy bats. The lyssavirus LBV has been isolated from the African straw-colored fruit bat (*Eidolon helvum*), the second of the two species in this African genus in various countries of Africa [21]. We isolated Ife virus and an alphaherpesvirus from the Malagasy straw-colored fruit bat [22]. These two viral species have also been detected from African straw-colored fruit bat [22, 23]. Therefore, we highly suspected the presence of LBV in Madagascar. Consequently, postexposure rabies vaccination should be provided after an exposure to Malagasy bats. However, people should keep in mind that rabies vaccine is less efficient against lyssavirus belonging to the phylogroup 2, including LBV [24].

We recently showed that a heminested PCR targeting a conserved region of the polymerase genes of lyssaviruses and applied to antemortem or postmortem skin biopsy (a specimen easier to collect than a piece of brain) was a successful

Table 2: Positive predictive values according to some characteristics of dogs tested for rabies (reported alone), Madagascar, 2006–2010.

Characteristics		Rabies laboratory results		Positive predictive values
		Negative	Positive	(%)
Suspected of rabies ($n = 257$)	Yes	74	114	60.6
	No	59	10	14.5
Responsible for bite ($n = 256$)	Yes	111	115	50.9
	No	21	9	30.0
Less than 4 years old ($n = 180$)	Yes	58	78	57.4
	No	33	11	25.0

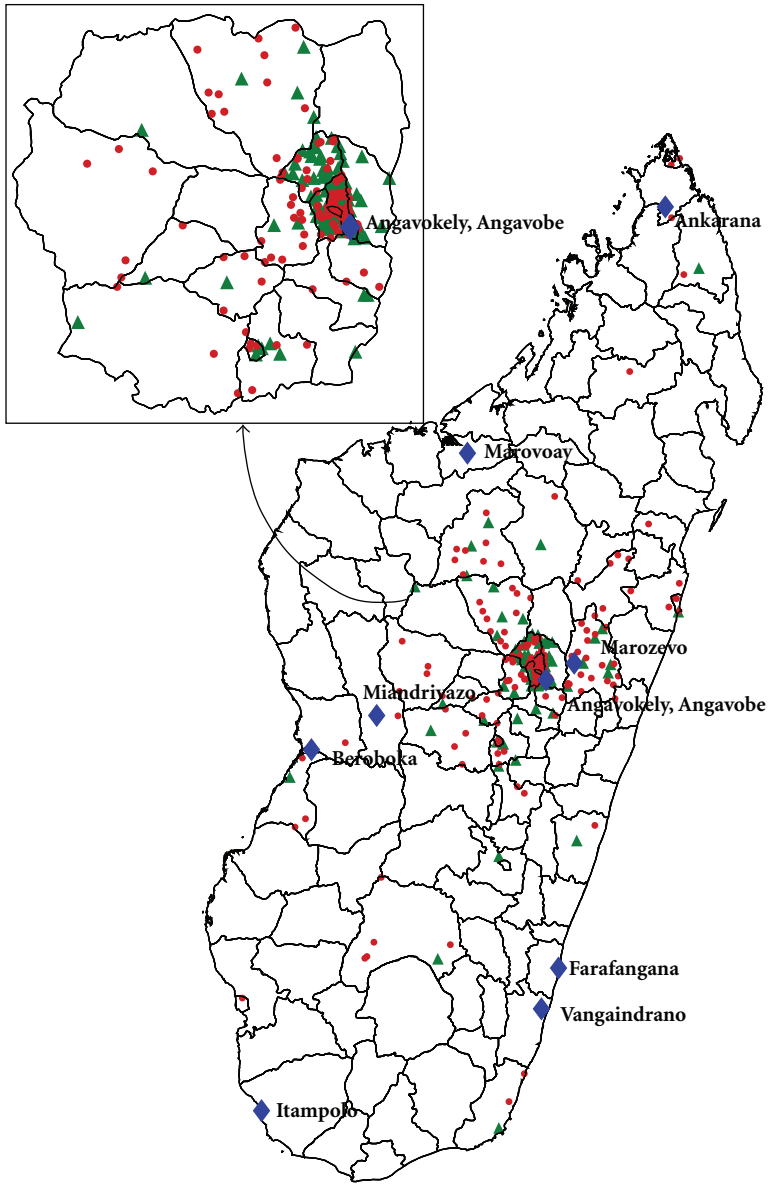

Figure 2: Distribution of the human and nonflying animal samples tested negative (green-filled triangle) and positive (red-filled circle) for rabies, and sites of bats sampling (blue-filled diamond) in Madagascar, 2005–2010.

Table 3: Bats samples tested for lyssavirus, according to the species and the site of capture, Madagascar 2005–2009.

Diet and bat Family	Species	Site of capture	No blood samples	No oral swabs
Insectivorous				
Hipposideridae	*Triaenops rufus*	Itampolo	18	0
Vespertilionidae	*Myotis goudoti*	Itampolo	1	0
	Miniopterus gleni	Itampolo	1	0
Molossidae	*Chaerephon pumilus*	Vangaindrano	22	0
	Mops leucostigma	Farafanga	14	0
		Vangaindrano	17	0
	Mormopterus jugularis	Itampolo	19	0
Frugivorous				
Pteropodidae	*Pteropus rufus*	Marovoay	130	104
		Marozevo	33	8
		Beroboka	29	0
		Miandrivazo	112	97
		Vangaindrano	38	32
		Angavobe	54	32
		Miandrivazo	2	2
	Eidolon dupreanum	2005–2009		
		Roost followup	753	465
		Angavobe and Angavokely		
Total			**1243**	**740**

procedure to perform rabies diagnostic [11]. We raised centres for postexposure prophylaxis staffs awareness of the performance of this procedure. Since that period (2008), we received postmortem skin biopsies from rabies-suspected cases, some of them coming far from Antananarivo, like Taolagnaro, on the south coast of the country (data not shown). Rabies infection was confirmed in 5 of these 6 cases. These samples easy to perform and to ship to the laboratory should be more promoted among health care personnel through Madagascar, to have a better idea of the prevalence of rabies in humans. Furthermore, this procedure should be also tested on carnivorous mammals, considering the sampling of skin carrying vibrissae (rich in nerve endings surrounding the base of these hairs). This method could help avoiding contamination of people sampling these animals by rabies virus-containing biological fluids and promote the sampling of rabies-suspected animals.

So far, for economic reasons, there are rabies postexposure prophylaxis centres in only 26 of the 111 administrative districts of Madagascar. We received samples of rabies-suspected cases from only 13 of them, and rabies virus circulation was confirmed in all of them. There is a need to confirm repeatedly its circulation in all of these 26 districts, especially in two islands (Nosy Be and Sainte Marie), where there is no recent report of rabid animals. Sampling should be promoted in the 13 other districts to evaluate the pertinence of these centres.

5. Conclusion

More than a century after the introduction of the vaccine against rabies in Madagascar, rabies remains endemic in the island. So far, preventing human rabies through dog rabies control and eventual elimination has been limited to local initiative. Madagascar, like other countries, is facing numerous public health issues. Because of the low incomes of the country and the lack of epidemiological data, this disease has not been prioritized, and a control program could not reasonably start. However, Madagascar is an island, and the elimination of rabies and its sustainability should be facilitated by the limited risk of introduction of rabid animals Therefore, the collection of such data (human and animal surveillance, dog ecology study, animal bites, etc.) should be promoted at first on pilot scale in order to validate the tools used. Afterward, data collection should be expanded to the rest of the country, while a pilot rabies control program (canine vaccination, canine population management, human postexposure prophylaxis, education, information, etc.) should start on pilot sites and then extended to the rest of the country.

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Research Article

Using Intradermal Rabies Vaccine to Boost Immunity in People with Low Rabies Antibody Levels

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Intradermal rabies vaccine is recommended by the World Health Organisation, but not all countries, including England, follow this recommendation. A group of 12 adults in England previously given pre-exposure intradermal rabies vaccine were considered to be non-immune to rabies because their rabies antibody titres were known to be less than 0.5 IU/mL. A cohort study examined the immunizing effect of increasing the participants' cumulative dose of intradermal rabies to 2.0 IU. All patients subsequently demonstrated rabies antibody levels >0.5 IU·mL supporting evidence of adequate sero-conversion. No adverse effects of intradermal rabies vaccine boosting were noted. Within the limits of a small study the findings support the hypothesis that adequate levels of rabies antibody can be achieved by a schedule of intradermal injections delivered on at least three occasions with a cumulative rabies vaccine dose of 2.0 IU.

1. Introduction

A study undertaken in 2007 on the duration of the immunogenicity of intradermal rabies vaccine demonstrated titres of rabies antibodies consistent with a protective response a decade or more after immunization with human diploid cell vaccine [1]. Twenty one of the 89 participants in that study failed to demonstrate titres of rabies antibodies greater than 0.5 IU/mL and were considered not to be adequately protected against rabies. Of the twenty one, none had received a cumulative dose of intradermal rabies vaccine greater than 1 IU nor had they received rabies intradermal vaccination on more than two occasions. Based on this observation, we hypothesised that the production of levels of rabies antibodies that can be correlated with protective efficacy requires a minimum cumulative dose of 2.0 IU of rabies vaccine administered intradermally over not less than three separate occasions. An antibody titre of greater than or equal to 0.5 IU/mL was considered indicative of seroconversion, providing an adequate titre, in line with the World Health Organization (WHO) recommendations [2].

The study reported in this paper was undertaken in order to examine that hypothesis.

2. Method

This study was based on inviting the 21 nonresponding participants of the first study to provide 4 mL blood samples to confirm the previously determined antibody titres. Twelve of the 21 were both willing and able to participate. They were given booster doses by the intradermal route to bring them up to a life time cumulative dose of 2.0 IU of rabies vaccine. In all cases this proved to require 1.0 IU of rabies vaccine. After an interval of approximately 6 weeks a second 4 mL blood sample was obtained. Antibody levels were measured from "blinded" blood samples to reduce the risk of bias. All serum samples were tested by the fluorescent antibody virus neutralisation test [3]. This test is regarded in the UK as the gold standard, with a high sensitivity for detecting postimmunisation antibody levels.

Prior to the study commencing approval was sought from the Health Protection Agency's Research Sponsorship Review Group, the West Yorkshire Primary Care Research and Development Unit for Research Governance Approval, the National Research Ethics Service for ethical approval and the Medicines and Healthcare products Regulatory Agency for a clinical trial authorization. This approval process commenced in August 2008 and concluded in March 2009. The Eudract Number granted was 2008-005465-56.

Following the protocol developed for this study all 21 possible participants were sent a postal invitation to participate in this study. This invitation informed them of the purpose of the study and what was expected of them. A form was included on which the possible participant was asked to indicate if they wished to discuss taking part and asking them to provide their preferred contact details. This form could be returned in an accompanying stamped addressed envelope. Returned forms were followed up with a telephone call made by one of the authors to confirm that the participant was still eligible and willing to participate. Arrangements to collect the blood samples and to give the booster dose of rabies vaccine were agreed by telephone and followed up in writing. In most cases the work was undertaken at the Leeds Overseas Travellers Clinic, Yorkshire, UK, where the participant had received their original course of pre-exposure rabies vaccine prior to travel.

At the first of the two clinic visits the participant was again advised about the study and asked to sign a consent form. A 4 mL venous blood sample was taken and collected in a plain glass tube and stored at 4°C. Two separate 0.5 IU doses of rabies vaccine were then administered by the intradermal route over the left deltoid muscle, the injections being between 2 and 3 cms apart. The vaccine used was Verorab (Pasteur Merieux) Lot number B0529, due to expire in May 2010. This vaccine is recommended for intradermal use by WHO [4]. Verorab is prepared using a cell culture technique using cells originally harvested from African Green Monkeys. All blood was collected by one of the authors who also gave all the intradermal immunizations to ensure consistency of technique. After an interval of six to eight weeks participants were seen again. Enquiries were made about any possible side effects from the immunization, and a second 4 mL venous blood sample was collected. Antibody results were matched against the list of participants to allow tabulation and review.

3. Subjects and Recruitment

The only people initially eligible to participate were the 21 participants from the previous study who had demonstrated antibody titres less than 0.5 IU/mL. Five people did not reply to the letter of invitation and could not be traced. Two had contraindications to participation in the form of additional intramuscular doses of rabies vaccine to protect them during travel to rabies risk areas during the two years since the first study. One had developed a serious illness and was now taking long-term steroids. Only one previous participant expressly refused to participate in this study.

Twelve previous participants agreed to take part again and on questioning did not appear to have any contraindication from doing so. No financial inducement was paid to the participants. All participants were, however, assured that if any problem did arise as a result of their participation there was a helpline telephone number to call. They were also informed in writing that the researchers were indemnified should there be any serious untoward consequences from participation. The eligibility criteria used were participation in the previous study, having a documented antibody titre of less than 0.5 IU/mL, not having received any rabies vaccine by any route since the first study, and not having any condition that might impair immunity.

4. Outcome Measures

The main outcome measure sought was the rabies antibody titre following the booster dose of rabies vaccine. A secondary outcome measure was the degree of change in antibody titre since the previous study. Participants were also all asked if they experienced any untoward effects of the booster dose.

5. Statistical Analysis

Laboratory results were tabulated. The key determination was the proportion of participants who, following booster intradermal rabies vaccine demonstrated antibody titres equal to or greater than 0.5 IU/mL. The mean rise in antibody titre was also determined and examined for the effects of age or gender.

6. Results and Discussion

The key results are displayed in Table 1.

Of the 12 participants the majority (75%) were women. Ages ranged from 20 to 71 years of age. All had received a total of 0.4 mL of previously available vaccines which is equivalent to 1 IU of rabies vaccine. Six of the 12 participants had received their previous rabies vaccine at a single clinic visit, five on two clinic visits, and one on four clinic visits. As shown in Table 1 pre-booster antibody titres are consistent with the previous estimation two years earlier. All participants demonstrated postbooster antibody titres higher than the minimum considered consistent with immunity to rabies. The mean pre-booster titre was 0.18 (CI 0.12–0.25), and the mean postbooster titre was 17.33 (CI1.48–33.19). The mean antibody rise was 17.15 IU/mL. The range of antibody rises was very wide, varying from 1.75 to 69.93 IU/mL. The 95% confidence intervals are 1.326–32.973 IU/mL.

The two results with increases in antibody titre of almost 70 skew the results and lie outside the 95% confidence intervals. Using a log transformation of the difference between pre- and posttitre levels gives a highly significant P value of <.01; the post-booster titre levels were significantly larger than the pre-booster titre levels (see Figure 1).

Table 1: Summary of the results obtained for each participant.

Participant number	Age	Gender	Year last immunised	Vaccine administered on days	Antibody titre in 2007	Pre-booster titre, 2009	Post-booster titre, 2009	Rise in antibody titre following immunisation
1	38	F	2003	0, 54	0.38	0.13	7.79	7.66
2	66	F	2000	0, 28	0.38	0.29	3.42	3.13
3	32	F	2002	0	0.06	0.07	5.92	5.85
4	67	M	2004	0	0.29	0.07	3.42	3.35
5	71	M	2003	0, 28	0.38	0.22	1.97	1.75
6	45	F	1998	0, 28	0.22	0.13	2.60	2.47
7	36	F	2003	0	0.38	0.29	13.5	13.21
8	30	F	2004	0	0.29	0.22	70.15	69.93
9	39	F	2005	0, 28	0.38	0.38	70.15	69.77
10	34	F	2002	0, 7, 21, 28	0.38	0.13	7.79	7.66
11	20	M	1999	0	0.38	0.17	7.79	7.62
12	28	F	2005	0	0.38	0.10	13.5	13.4

All titre results expressed in IU/mL.

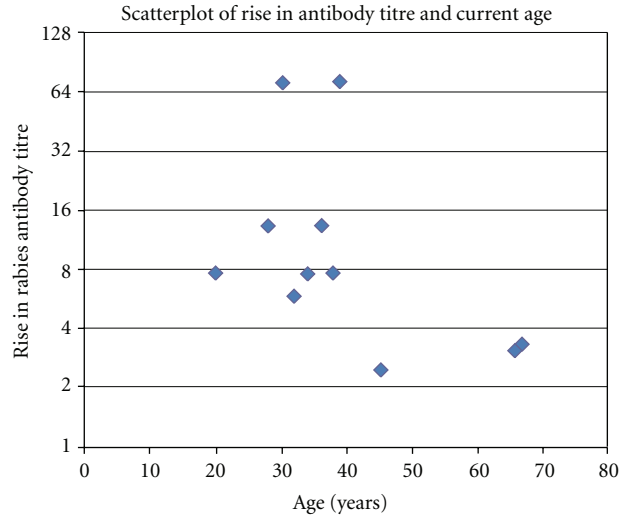

Figure 1: Scatter plot of antibody titre rise and age in years.

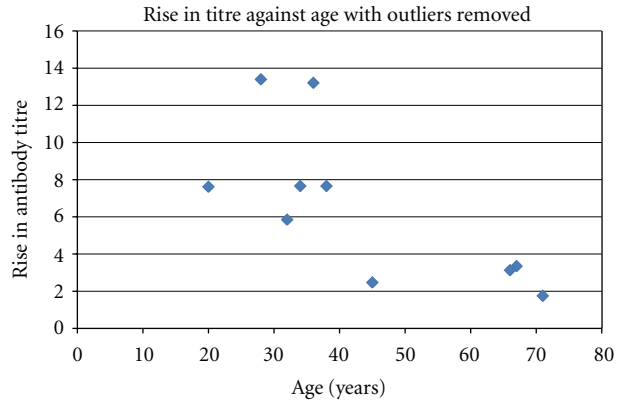

Figure 2: Scatter plot of rise in tire after boosting with outliers removed against age.

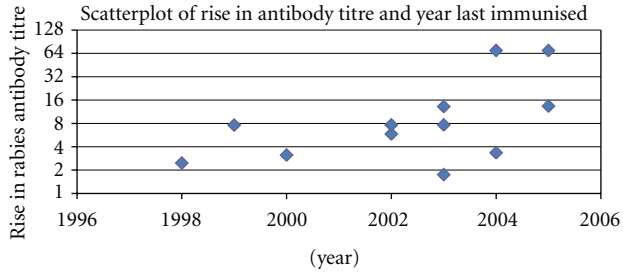

Figure 3: Scatter plot of rise in antibody titre and year last immunized.

The most notable increases in antibody titre were observed in two women in their 30 s who had received their last rabies immunization between 4 and 5 years previously. Apart from age and gender no explanation for their high antibody response was determined. If these two results are excluded, the results did not show any obvious gender difference.

The apparent decline in antibody response with age is shown even if the two outliers are removed from the analysis as shown in Figure 2.

Figure 3 suggests that there was a decline in antibody response after 10 years. The titre immediately prior to boosting did not appear to have any direct effect on the titre achieved after boosting immunization. This can be observed in Figure 4.

Participant 2 disclosed after the second blood sample that she had received chemotherapy for breast cancer since the previous study, if this had been disclosed earlier, she would have been excluded from the study. Interestingly, she demonstrated a good antibody response to the intradermal immunization. None of the participants reported any noticeable side effects from the immunization. None of the participants called the telephone helpline offered at entry to the study. Rabies remains a serious global challenge with an estimated 55,000 deaths each year [5]. The availability of

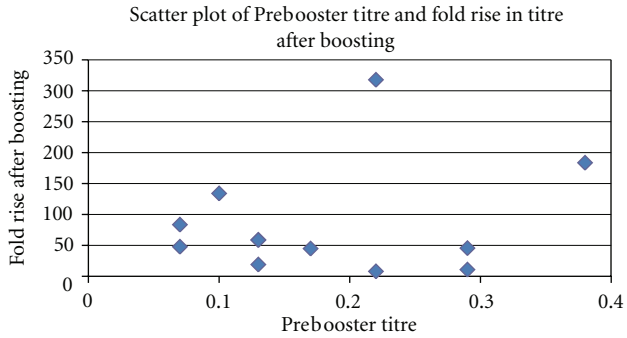

Figure 4: Scatter plot of pre-booster tire and fold rise in titre after boosting.

an effective vaccine is restricted by total vaccine production and relatively high cost. If intradermal rabies immunization schedules were introduced for pre-exposure prophylaxis, it would both increase the global supply of vaccine doses and reduce the cost per person immunised.

It is not possible to accurately determine the level of rabies virus neutralizing antibody adequate to provide protective immunity for humans. The World Health Organization regards 0.5 IU/mL as an adequate level of antibody after vaccination [2] and that has been accepted by the authors of this paper, providing the basis for our confidence that 12 previously inadequately protected individuals now have adequate protective antibody levels. Previous work undertaken by the authors suggests that this protection should last for at least ten years [1].

Versions of rabies vaccine previously used on the patients in this study contained 2.5 IU/mL. Many previous reports that discuss the effectiveness of both intramuscular and intradermal vaccines quote the dose administered in volume terms. The vaccine available for this study, Verorab, has twice the concentration containing 5 IU/mL, which is supplied in 0.5 mL vials each containing 2.5 IU of rabies vaccine. In order to avoid confusion this paper has quoted the dose in International Units rather than volume administered.

There were only 12 participants in this study so there is a possibility that they do not represent the true population of responders to boosting with intradermal vaccine. Calculation of the 95% confidence intervals of a small sample with a skewed distribution results, as in this case, in wide confidence intervals. Participants ranged widely in age and in time since previous rabies immunization. All developed an effective rabies antibody response. All 12 participants in this study demonstrated a good response to the booster doses supporting the hypothesis that antibody levels consistent with immunity to rabies are likely to be achieved if a total of 2 IU of rabies vaccine have been administered over three or more occasions. In this study the last dose had been administered within the previous 10 years. Taken with the findings of long-lasting immunity following intradermal rabies vaccine in our earlier study [1], there is evidence to support a pre-exposure intradermal rabies immunization schedule based on delivering 2.0 IU spread over three doses. The interval between these three initial doses could be based on the observations of Thai studies which demonstrated effective protection with doses given on days 0, 7, and 28 described by Strady et al. [6] who used the intramuscular route and Kamoltham et al. [7] who used purified chick embryo cell vaccine given intradermally. A study by Naraporn et al. [8] examined the immune response to rabies booster immunization after an interval of 5 years and showed that all 36 patients who completed the study at 28 days had a good anamnestic antibody response to two intradermal booster injections of purified duck embryo cell vaccine given 3 days apart. Malerczyk et al. [9] report a study in 15 German veterinarians who had received purified chick cell embryo cell vaccine 14 years previously who were boosted with intramuscular purified chick cell embryo vaccine. All ten veterinarians who submitted blood samples after immunization demonstrated a good anamnestic response. Suwansrinon et al. [10] describe a study in Thailand in which 53 patients who had received rabies immunization between 10 and 20 years previously were given two 0.9 IU doses of Vero cell rabies vaccine three days apart. Two weeks after immunization all had antibody levels that exceeded the critical threshold considered to provide adequate immunity of 0.5 IU/mL.

The use of intradermal human diploid cell rabies vaccine for boosting purposes was examined in a study undertaken in 1987 which followed up 40 laboratory workers who had been given intradermal rabies vaccine in 3 separate doses totaling 0.75 IU [11]. Twenty of these workers demonstrated titres considered to be protective at 1 year, but by 2 years 5 were considered to have unprotective levels. Intradermal boosters given to 4 of these 5 laboratory workers produced high titres of rabies antibody. That study recommended serological testing every two years with a booster dose given to those with what are regarded as unprotective titre levels.

The observations of the study being reported in this report suggest that increasing the initial course to a total of 2 IU given over three clinic visits will provide effective rabies protection. Review of the literature and our previous study suggest that adequate immunity will be maintained for at least 10 years without the need for expensive serological testing or boosting. It is reasonable to conclude that immunization with at least 2 IU of rabies vaccine by the intradermal route should result in an antibody titre that will provide protection. This regimen could preserve the limited stocks of rabies biologicals, including rabies immunoglobulin. It is suggested that the time intervals used between doses can be based on the work of Strady et al. [6], with an initial dose of 1 IU on day 0 followed by 0.5 IU on days 7 and 28. These doses are relatively easy to translate into volume terms based on the concentration of the vaccine available for use.

No adverse effects were reported after immunization supporting the hypothesis that boosters of two intradermal 0.5 IU Vero cell-derived rabies vaccine injections can safely be coadministered after 10 years with immunity maintained at an adequate level.

This study did not examine the effect of giving a single 0.5 IU dose of rabies vaccine as a 10-yearly booster; however the good level of response suggests that further cost and vaccine savings could be safely achieved. Further research to

assess the increase in antibody titre following a single 0.5 IU dose of rabies vaccine is advisable. The absence of any serious side effects in this small sample of 12 patients given intradermal rabies vaccine is reassuring and helps to give confidence that the intradermal immunization route is not only effective but also safe [5].

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Conflict of Interests

The authors have declared that no competing interests exist.

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Research Article

Immunogenicity of Simulated PCECV Postexposure Booster Doses 1, 3, and 5 Years after 2-Dose and 3-Dose Primary Rabies Vaccination in Schoolchildren

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Objectives. To assess the immunogenicity of intradermal (ID) booster doses of Purified Chick Embryo Cell rabies vaccine (PCECV, Rabipur) administered to Thai schoolchildren one, three and five years after a primary ID pre-exposure (PrEP) vaccination series. *Methods*. In this follow-up study of a randomized, open-label, phase II clinical trial, two simulated post-exposure booster doses of PCECV were administered on days 0 and 3 intradermally to 703 healthy schoolchildren, one, three or five years after primary vaccination with either two or three ID doses of 0.1 mL PCECV. Blood was drawn immediately before and 7, 14 and 365 days after the first booster dose to determine rabies virus neutralizing antibody (RVNA) concentrations. *Results*. An anamnestic response of approximately 30-fold increase in RVNA concentrations was demonstrated within 14 days after booster. All children (100%) developed adequate RVNA concentrations above 0.5 IU/mL. No vaccine related serious adverse events were seen in any of the vaccinees. *Conclusion*. ID rabies PrEP with PCECV is safe and immunogenic in schoolchildren and the anamnestic response to a two booster dose vaccination series was found to be adequate one, three, and five years after a two- or three-dose primary PrEP vaccination series.

1. Introduction

Rabies post-exposure prophylaxis (PEP) after an exposure to a rabid animal has been demonstrated to be efficacious using tissue culture vaccines (TCV) including purified chick embryo cell vaccine (PCECV), administered either intramuscularly (IM) or intradermally (ID) [1, 2]. However, human rabies remains a significant health problem in countries of Asia and Africa, where more than 99% of the exposures come from rabies-infected dogs that inhabit rural and urban areas. The vast majority of the estimated 55,000 human deaths that occur worldwide every year occur on these two continents [3, 4], mainly due to lack of awareness that results in delayed, inadequate PEP, or even no PEP administered to patients exposed to rabid animals. A significant number of bite exposures and rabies cases occur in children under 15 years of age [5–8]. It has been reported that in Thailand by the age of 15 years approximately one-third of all children will have experienced a dog bite, indicating the potential risk for children to be exposed to a rabid animal [9]. While PEP clearly saves lives, human rabies cases, especially in children, continue to occur despite the availability of vaccines and biologicals. Almost all of these human rabies cases could have been prevented, and almost all occurred due to a lack of receiving PEP. One possible alternative to making sure that every child received adequate PEP after exposure is to administer pre-exposure prophylaxis (PrEP) to those living in high-risk regions. The use of PrEP in children living in

Table 1: Number and percentage of children reaching adequate RVNA concentrations (≥0.5 IU/mL) after administration of simulated post-exposure booster doses, 1, 3, or 5 years after two or three primary vaccination doses, as determined by RFFIT.

Group	1-year				3-year				5-year			
	pre	D7	D14	D365	pre	D7	D14	D365	Pre	D7	D14	D365
2d	(6/84)	(81/84)	(81/81)	(51/77)	(4/48)	(35/48)	(47/47)	(24/41)	(10/82)	(75/82)	(79/79)	(29/57)
%	7%	96%	100%	66%	8%	73%	100%	59%	12%	91%	100%	51%
3d	(22/63)	(61/61)	(58/58)	(55/59)	(24/60)	(58/60)	(57/57)	(45/52)	(41/89)	(88/89)	(85/85)	(59/62)
%	35%	100%	100%	93%	40%	97%	100%	87%	46%	99%	100%	95%

2d: two-dose primary vaccination; 3d: three-dose primary vaccination; pre: before booster.

areas of high risk of exposure to rabies would reduce the number of vaccine booster doses required and eliminate the need to administer rabies immunoglobulin (RIG) after an exposure has occurred. For example, persons that have been vaccinated previously with a tissue culture rabies vaccine and are subsequently exposed to a rabid animal only require two booster doses of vaccine, administered on days 0 and 3, either IM or ID [4]. Previous reports have demonstrated that PCECV is immunogenic and safe when given intradermally [10–12]. Recent studies from Thailand and India revealed that the current WHO PrEP recommendations of three IM or ID doses are adequate in schoolchildren [13, 14] and toddlers [15]. A study using PCECV in toddlers administered concomitantly with Japanese encephalitis vaccine (JEV) demonstrated adequate tolerability and immunogenicity of both vaccines and indicated the suitability of introducing rabies vaccine into the Expanded Program on Immunization (EPI) schedule. In addition, a study with purified verocell rabies vaccine (PVRV) was conducted in infants, indicating adequate immune responses when rabies vaccine was administered concomitantly with pediatric routine combination vaccine (diphtheria, tetanus, whole cell pertussis, inactivated poliomyelitis; DTP-IPV) [16]. However, when infant or preschool rabies vaccinations are missed, vaccination in early school-age children could be a practical and efficient solution to protect this most vulnerable population against rabies. In this study we investigated whether two or three ID doses of PCECV would be immunogenic in children and concluded that the current recommendation of three doses given ID is appropriate [13]. The study population, clinical trial design, and results of the primary vaccination have been published earlier [13].

2. Methods

2.1. Clinical Trial. In this long-term followup, the anamnestic response of Thai schoolchildren that received two (simulated) post-exposure booster doses of PCECV was investigated up to five years after the primary vaccination PrEP series was administered. Details of the study conduct have been described earlier [13]. Briefly, subjects enrolled in the clinical trial included healthy schoolchildren, aged 5 to 8 at the time that the primary vaccination with two or three 0.1 mL ID doses of PCECV was administered. Subjects were followed for one, three, or five years after primary PrEP and then received two ID booster doses of 0.1 mL PCECV on days 0 and 3, simulating the current recommended PEP booster recommendations, that is, administering the 2-dose booster doses, without RIG, as if an exposure had occurred. The PCECV used for the primary vaccination series and for the 1-year and 3-year booster doses was Novartis Vaccines' Rabipur, produced in India; batch no. 725 (potency 7.25 IU/mL). For the 5-year group, batch no. 1471 (potency 9.81 IU/mL) was used. The objectives of the study were to demonstrate long-term postbooster rabies virus-neutralizing antibody (RVNA) protection, defined as RFFIT antibody concentrations ≥0.5 IU/mL, one, three, and five years after the primary vaccination, to evaluate whether adequate RVNA concentrations is achieved in all subjects and to compare the immune responses of the 2-dose versus 3-dose ID regimen of PCECV. This study was conducted under the auspices of the Ministry of Public Health, Thailand, following the research principles set out in the Declaration of Helsinki and Good Clinical Practice guidelines. Approval of the study protocol was obtained by the Ethical Review Committee for Research in Human Subjects, Ministry of Health; all parents and legal guardians of subjects were informed of the study protocol prior to enrollment, and written informed consent was obtained from parents or legal guardians of all subjects prior to enrollment. The study was registered at ClinicalTrials.gov (identifier: NCT01107275). A flow diagram of study participants as suggested in the CONSORT Statement is given in Figure 1.

2.2. Serology. Blood was drawn before administration of the first of two booster doses and on days 7, 14, and 365 days later. Serology testing was performed in the same laboratory as in the first part of the study, (Queen Saovabha Memorial Institute, Bangkok, Thailand) for determination of RVNA concentrations, using the rapid fluorescent focus inhibition test (RFFIT), as described earlier [17].

3. Results

One year after the primary vaccination, RVNA concentrations had decreased (Figure 2(a)) with 7% and 35% of the vaccinees still having adequate RVNA concentrations above 0.5 IU/mL, in the 2-dose and 3-dose group, respectively, (Table 1). This percentage of subjects with adequate RVNA concentrations did not change significantly over time (Figures 2(b) and 2(c)); 8% and 40% of subjects in the 3-year group and 12% and 46% of subjects in the 5-year group, respectively, maintained adequate RVNA concentrations (Table 1). After receiving two booster doses of PCECV, on

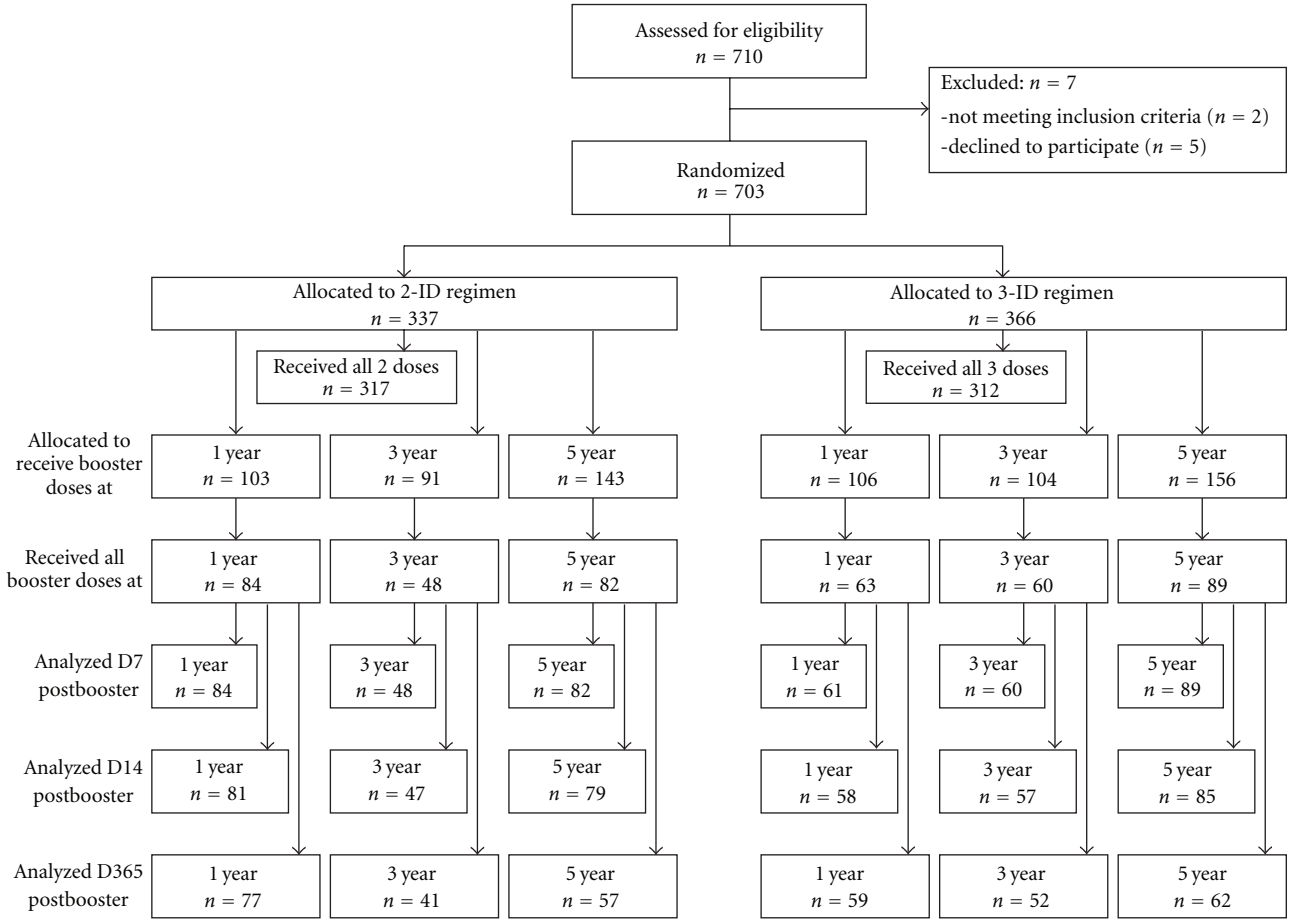

Figure 1: Flow diagram of study participants (according to CONSORT Statement).

day 0 and day 3, RVNA concentrations increased significantly in all study groups, thus eliciting adequate RVNA concentrations on day 7 postbooster in 100%, 97%, and 99% of the children in the 3-dose groups, and 96%, 73%, and 91% of the children in the 2-dose group, at one, three, and five years after primary vaccination, respectively. By day 14, every child (100%) had reached adequate RVNA concentrations, regardless of the time interval between primary vaccination and booster or whether having received two or three primary doses (Table 1). Thus the objective was met to demonstrate long-term postbooster RVNA protection, defined as RFFIT antibody concentrations ≥0.5 IU/mL, 1, 3, and 5 years after the primary vaccination, as well as to demonstrate that adequate RVNA concentrations are achieved in all subjects. Fourteen days after booster, the 2-dose regimen proved equivalent to the 3-dose regimen in eliciting adequate response (100% adequate RVNA concentrations in all groups), while on day 7 after booster, the percentage was lower in the 2-dose group. When comparing actual RVNA concentrations, GMCs were about 3-fold higher in the 3-dose group than in the 2-dose group. This difference was seen throughout the study (Figure 2).

4. Discussion

When a person has been previously immunized with a PrEP series of three doses of rabies vaccine, the current recommendations for PEP include the administration of two booster doses of a WHO-recommended tissue culture vaccine. It is neither necessary nor recommended to administer RIG to individuals that have received a tissue culture vaccine previously. The question as to whether the time interval between primary vaccination series and the PEP booster series following an exposure has an influence on the ability of a patient to elicit an anamnestic response is an important concern for public health officials that may be considering the use of PrEP to protect populations living in areas with a high risk of exposure to rabies. In this study we investigated the anamnestic response in subjects that had received a two booster dose series of PEP one, three, and five years after the primary PrEP immunization, and we have confirmed that an adequate and rapid immune response occurred in all subjects.

Interestingly, RVNA concentrations and the percentage of patients that produced adequate titers did not change significantly over the years. In subjects that had been

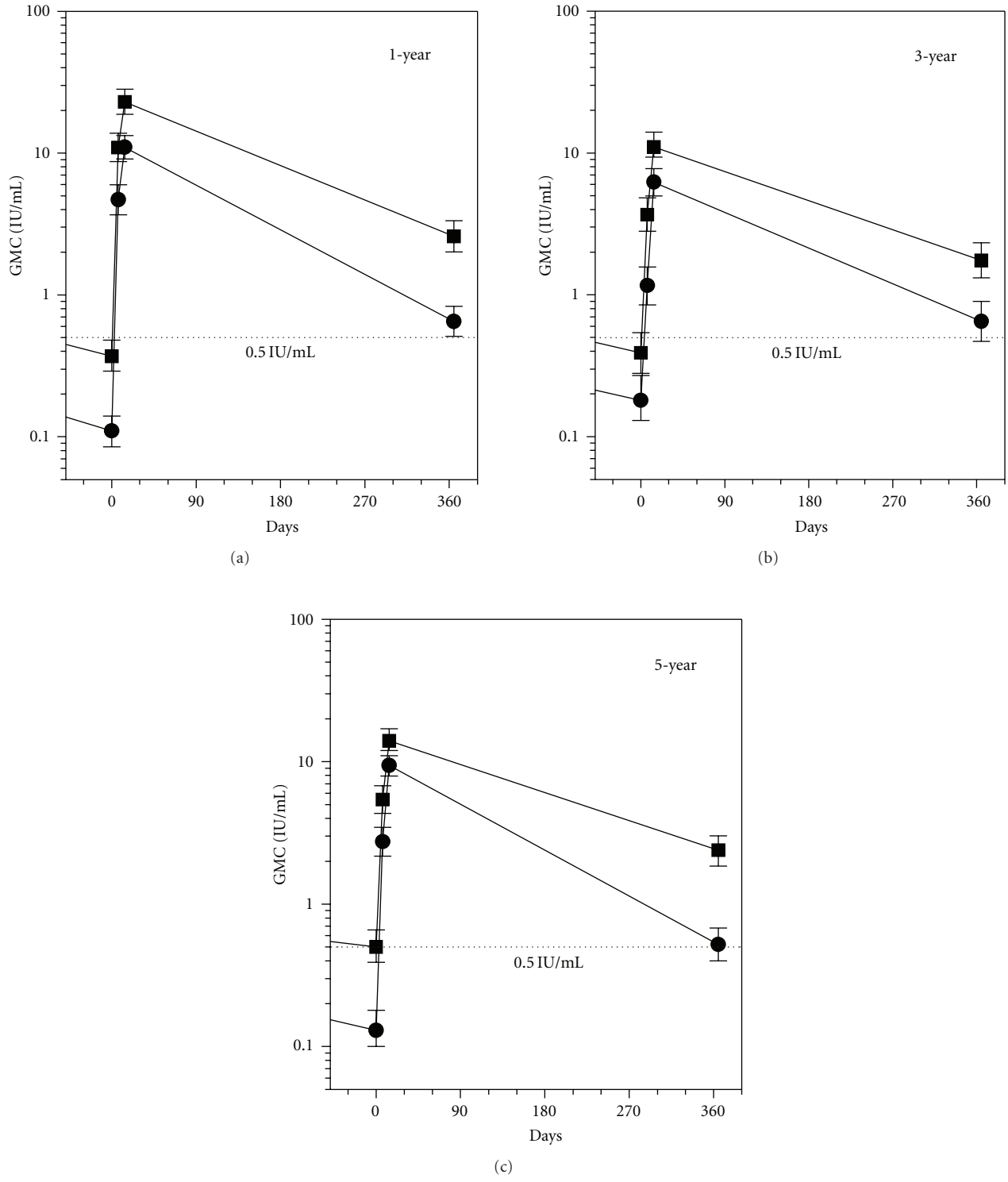

Figure 2: Immune response after two simulated post-exposure intradermal 0.1 mL booster doses of PCECV on days 0 and 3, administered one (a), three (b), or five years (c) after completion of a primary vaccination series. ●: 2 ID doses; ■: 3 ID doses; error bars represent 95% confidence intervals · · · : RVNA concentrations regarded as adequate for protection. (0.5 IU/mL).

vaccinated five years previously, approximately the same RVNA concentrations were observed as in subjects that had been vaccinated one and three years earlier. After the two-booster dose PEP series, a comparable immune response was observed in all subjects regardless of the time elapsed since their initial PrEP series. A more relevant consideration is how many doses were included in the initial primary vaccination series: those subjects that received a three-dose primary

PrEP series had higher levels of RVNA concentrations and higher booster responses than subjects that received only a two-dose primary PrEP series (Figure 2). However, although GMTs of RVNA concentrations in the group that received a two-dose PrEP series were significantly lower throughout the study, in this group all subjects achieved adequate RVNA concentrations above 0.5 IU/mL, when two booster doses were given up to five years after primary vaccination. The fact that all subjects reached adequate RVNA concentrations by day 14, regardless of the time interval between primary series, and booster doses or the number of doses in the primary series is reassuring. However, the overall lower RVNA concentrations in the 2-dose group resulted in a lower percentage of adequate RVNA concentrations on day 7. In particular, in the 2-dose group adequate immune responses were only seen in 73% of children (3-year data), compared to 97–100% in the 3-dose groups. This leaves a vulnerable period of a few days in more than few subjects after a 2-dose primary vaccination series. Whether this would lead to treatment failure and development of rabies remains questionable. In PEP of previously unvaccinated subjects, adequate RVNA concentrations do not develop before day 14 either. Clearly here RIG is recommended to cover the lag period. However, in reality RIG is only administered in 2 to 10% of all cases, where it would be indicated [18], and treatment failures are seen extremely rarely. To be on the safe side, however, as administration of RIG is not considered necessary or recommended for previously vaccinated subjects, a 3-dose primary vaccination regimen might be considered more suitable for individual protection.

Additionally the question how to prove previous vaccination has to be discussed. It is not uncommon that children or parents forget about the vaccines that they had been given. A serologic testing may not be a suitable method for proof of earlier vaccination. Such testing may not be available everywhere, is quite expensive, and—most critically—would provide results too late for a decision whether to give booster doses without RIG or whether to start a complete series of PEP, including RIG when indicated. Therefore, a system of documentation of each vaccination in a booklet is preferred. As a matter of fact, in absence of documented proof of vaccination, a full PEP course including administration of RIG would be required.

The WHO recommends that diagnostic laboratory workers, rabies researchers, and other people at continuous risk (where rabies virus is present continuously, often in high concentrations, and where specific exposures to rabies are likely to go unrecognized) should have their serological titers evaluated every six months for the presence of RVNA and receive a single booster vaccination when their RVNA concentrations fall below 0.5 IU/mL [4]. For the general population living in endemic countries, it is sufficient to receive a routine ID booster series with 0.1 mL of PCECV without routine serology testing, which is expensive and difficult to perform. Due to the fact that immune memory is established in persons that have been vaccinated with a TCV, an anamnestic immune response is induced after a PEP-booster series using 0.1 mL of a TCV (PCECV) ID booster doses, as demonstrated in this study up to five years after completion of the primary vaccination.

The results of this study are in line with results from another study investigating abbreviated and less doses intradermal pre-exposure vaccination schedules. In one of the study arms, Khawplod and coworkers administered two ID doses at two sites on a single visit as primary vaccination, using PCECV or PVRV. Upon two ID booster doses (Day 0 and 3) one year later, all subjects elicited anamnestic immune responses and adequate RVNA concentrations [19].

A striking additional finding in our study was that 12 of 703 children (1.7%) were actually exposed to rabies by potentially rabid animals during the study period. These were given appropriate PEP as predefined in the study protocol, and they were further excluded from serology analyses but were followed for a period of one year. All remained healthy during the observation period. The high number of exposures clearly shows that rabies is an endemic threat to children in Thailand.

5. Conclusion

While the current recommendation of PrEP vaccination consists of three doses of rabies vaccine administered ID or IM [4], a PrEP vaccination series using two or three doses of 0.1 mL PCECV administered ID is safe and immunogenic in school children, and anamnestic responses occurred in all subjects after two booster doses were administered up to five years later. This indicates that when an exposure occurs, two booster doses of vaccine administered ID three days apart may be appropriate in previously immunized persons that may have received only two initial doses of a PrEP series although three initial doses lead to higher immune responses and longer lasting protection. Reduced PrEP regimens would reduce the cost of protecting vulnerable populations against rabies and would promote better compliance, thus supporting opportunities to conduct mass PrEP rabies vaccination in children, the population most at risk of dying of this dreaded disease.

Conflict of Interests

Drs. Gerlind Anders and Claudius Malerczyk are full-time employees of Novartis Vaccines and Diagnostics. Dr. Thavatchai Kamoltham has received travel grants from Novartis Vaccines and Diagnostics.

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Research Article

Immunogenicity Studies in Carnivores Using a Rabies Virus Construct with a Site-Directed Deletion in the Phosphoprotein

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Different approaches have been applied to develop highly attenuated rabies virus vaccines for oral vaccination of mesocarnivores. One prototype vaccine construct is SAD dIND1, which contains a deletion in the P-gene severely limiting the inhibition of type-1 interferon induction. Immunogenicity studies in foxes and skunks were undertaken to investigate whether this highly attenuated vaccine would be more immunogenic than the parental SAD B19 vaccine strain. In foxes, it was demonstrated that SAD dIND1 protected the animals against a rabies infection after a single oral dose, although virus neutralizing antibody titres were lower than in foxes orally vaccinated with the SAD B19 virus as observed in previous experiments. In contrast, skunks receiving $10^{7.5}$ FFU SAD dIND1 did not develop virus neutralizing antibodies and were not protected against a subsequent rabies infection.

1. Introduction

The European rabies landscape has changed notably in the last 30 years as a result of oral vaccination of foxes (*Vulpes vulpes*) against rabies. Many countries in West and Central Europe eliminated terrestrial wildlife rabies by distributing oral rabies vaccine (ORV) baits. The red fox is no longer the sole target species for those baits; oral rabies vaccination programmes are targeted at many different animal species in many different regions, worldwide. The presently available commercial ORV are all replication-competent viruses and therefore pose an intrinsic safety risk. Vaccine virus-associated rabies cases have been reported in target and nontarget species [1–3]. Also, adverse reactions have been observed in humans after direct exposure to a recombinant ORV [4, 5]. Furthermore, several animal species are difficult to vaccinate against rabies by the oral route using the available products. Hence, an ongoing programme for safer ORV candidates with improved efficacy has been initiated since the first ORVs became commercially available [6]. One group of candidates is based on site-directed mutations of the rabies virus genome. Originally, these efforts focused on the gene encoding the rabies virus glycoprotein. The principal modality of protection against rabies virus is the generation of virus-neutralising antibodies (VNA) against the glycoprotein [6]. However, other facets of innate and adaptive immunity also contribute to virus clearance. Moreover, the role of cell-mediated and humoral

immunity against the nucleoprotein, matrix protein, and phosphoprotein in the protection of animals against rabies remains unclear. For these reasons, other rabies virus genes offer possibilities for the development of highly attenuated viruses, for example, the ability of the phosphoprotein to counteract transcriptional activation of interferon (IFN) Type 1 by interfering with the phosphorylation of IRF-3 [7, 8]. A site-directed deletion of a small internal domain (aa 176–186) of the phosphoprotein abolishes the ability to efficiently prevent IRF-3 activation and thereby preventing IFN Type 1 induction [9]. A virus construct, SAD dIND1, was developed comprising the aa 176–181 deletion in the phosphoprotein that had lost most of its inhibitory activity in preventing IRF-3 activation. This construct was shown to be completely avirulent in adult mice after i.c. administration [9], underscoring the type I IFN system as probably the most powerful antiviral response capable of controlling viral infections in the absence of adaptive immunity [10]. A pilot experiment in two wildlife reservoir species was initiated to investigate whether SAD dIND1 is more immunogenic than the parental strain SAD B19. The two wildlife reservoir species assessed included the highly susceptible red fox and the striped skunk (*Mephitis mephitis*); the latter is an animal species notoriously refractory to oral rabies vaccination [11, 12]. Although foxes vaccinated with SAD dIND1 by the oral route survived a subsequent rabies challenge, the construct was not able to protect skunks against a subsequent rabies infection.

2. Material and Methods

2.1. Virus Construct. The SAD dIND1 construct was developed as previously described [9] using the recombinant rabies virus SAD L16 comprising the consensus sequence of the ORV strain SAD B19. Through site-directed mutagenesis codons specifying the amino acids 176 to 181 of the phosphoprotein were deleted. The virus material used in these studies was propagated using a MOI of 0.1 in BSR T7/5 cells, a BHK-derived cell line stably expressing t7 RNA polymerase [13]. SAD dIND1 is considered a genetically modified organism (GMO) and is classified as a BSL2 pathogen in Germany. The permits for the animal studies with such GMOs have been obtained from the appropriate regulatory authorities (AZ-66230-1501-3, Landesverwaltungsamt Sachsen-Anhalt, Germany).

2.2. Challenge Virus. The challenge virus, CVS/USA/TX Coyote/295/R/061893, was obtained from CDC (USA) and originally isolated from a salivary gland of a coyote (*Canis latrans*). The original challenge virus had been inoculated in a fox and reisolated from the brain after the animal succumbed to rabies. The virus isolate was passaged once in a fox and finally reisolated from the salivary gland. The foxes and skunks were administered 1.0 mL of the challenge virus ($10^{5.1}$ MICLD50) in the M. masseter by the i.m. route.

2.3. Animals. The captive bred animals were obtained from different commercial sources; foxes from Fa. Phu Foxpol, Poland, and skunks from AGHIA Birds Company, the Netherlands. The animals were identified by implantation of a microchip (UNO BV, Zevenaar, The Netherlands). Vaccinated foxes were kept individually in cages housed within an isolation unit of the animal house at IDT. Vaccinated skunks were caged in groups within an isolation unit until the time of challenge when each animal was housed individually. The control animals were housed in the outside animal enclosure until administration of the challenge virus when the animals were also transferred to individual cages within an isolation room. Foxes were fed once a day and water was offered ad libitum. Foxes received fresh commercial food for fur animals, (Schirmer & Partner, Doehlen [D]). The skunks were fed commercial dry pet food twice a day (100 g = 25 g cat food (Drei-Mix, Miehlitz KG) + 75 g dog food (Good deal, Voro-Dog Vertrieb, Enger), soaked with water substituted with 5 g vitamin mixture per animal once per day (Multivit, Inropharm GmbH Fürstenzell)). The skunks were also fed fresh fruit daily. The animals were observed at least once a day. After challenge, the animals were observed more frequently, and, on onset of CNS-related clinical signs, the animals were euthanized with T61 intracardial, 0.3 mL per body weight kilogram (Intervet Deutschland GmbH, Unterschleissheim [D]), after sedation with a ketamine 1.0–2.0 mL 10% (WDT eG, Garbsen [D])-xylazine [Xylariem, Riemser Arzneimittel, Riems [D]) mixture.

The housing conditions of the animals met the conditions as stated in the German animal welfare act §2&2a and the recommendations of the GV-SOLAS (Society for laboratory animal science). All experiments were undertaken in accordance with the German animal welfare act §8a, Abs. 1 and 2.f.

Table 1: Summary of the experimental design with SAD dIND1 in foxes and skunks (o.g.: oral gavage; i.m: intramuscularly).

Animal	Number	Dose FFU/mL	Route	Observation period (days)
Fox	3	$10^{6.3}$	o.g.	62
Fox	3	$10^{7.3}$	o.g.	62
Fox	2	$10^{6.3}$	i.m.	62
Skunk	3	$10^{7.5}$	o.g.	45

2.4. Vaccination. Eight adult foxes were vaccinated with 1.5 mL SAD dIND1 using different doses and routes of administration (Table 1). The animals were challenged together with 2 control animals 62 days after administration of SAD dIND1. Fifty days after challenge, all surviving animals were euthanized. Blood samples were taken on day 0 (prior to vaccination), 28, 58, and 112 after vaccination. During a dose-dependent study with different vaccine candidates, 3 skunks received $10^{7.5}$ FFU SAD dIND1 by direct oral instillation and were challenged 45 days later together with 2 control animals. Blood samples of the skunks were collected—5, 28, 42, and 57 days after vaccination. All animals were sedated during administration of SAD dIND1 and blood sampling.

2.5. Assays

2.5.1. FAT/IHC. The presence of rabies virus antigen in fox brain samples was examined using the Fluorescent Antibody Test (FAT) as described by Dean et al. [14]. Samples of hippocampus from skunks were fixed in 4% phosphate-buffered formaldehyde and processed for paraffin-wax embedding and immunohistochemistry (IHC), slightly modifying a method described previously [15]. The hippocampus of skunks has been shown to be highly suitable for these purposes [16].

2.5.2. RFFIT. Blood samples collected were examined for the presence of rabies VNA using the rapid fluorescent focus inhibition test (RFFIT) [17], with the modifications of that method as described by Cox and Schneider [18]. Prior to testing, serum samples were heat-inactivated for 30 minutes at 56°C. To calculate the titer, a 50% reduction in concentration of rabies virus in vitro was calculated by use of inverse interpolation (SAS software 9.2). The rabies VNA titers were converted to international units (IU) by comparison with international standard immunoglobulin adjusted to 0.5 U/mL, which served as a positive control.

3. Results

All foxes vaccinated with SAD dIND1 survived the lethal challenge irrespective of dose and route of administration and were euthanized 50 days after challenge. Meanwhile, both control animals were euthanized upon showing clinical signs to rabies infection 10 and 11 days after challenge. Rabies antigen was detected in brain material of both animals. All foxes receiving SAD dIND1 seroconverted with VNA titres greater than the arbitrary threshold of 0.5 IU/mL (Table 2). The level of rabies VNA antibodies indicated a dose-and route-dependent response. In contrast to the foxes, SAD dIND1 did not confer sufficient immunity in skunks to prevent infection after challenge exposure. Unfortunately, the small sample size of skunks was even further reduced. One skunk receiving SAD dIND1 was excluded from challenge because it developed a severe dermatitis during the observation period and was located to another isolation unit. All 4 remaining animals succumbed to rabies as confirmed by IHC. The two control animals and the two vaccinated animals were euthanized upon showing clinical signs of CNS disorders on day 13 after challenge. None of the skunks that received SAD dIND1 developed detectable levels of rabies VNA during the observation period (Table 3). After challenge, both control animals had detectable levels of VNA, meanwhile both animals that received SAD dIND1 remained seronegative.

Table 2: The titre of virus neutralizing antibodies measured in the fox blood samples by RFFIT are expressed in IU/mL. The final blood sample (B3) was taken 50 days after challenge (GMT; geometric mean titre; o.g: oral gavage; i.m: intramuscularly).

Animal	Route	Dose FFU/mL (10 log)	B1 (day 28)	B2 (day 58)	B3 (day 112)
7879	o.g.	7.3	47.5	23.8	80.0
0200	o.g.	7.3	11.9	47.5	80.0
9787	o.g.	7.3	56.6	11.9	47.5
	GMT		31.7	23.8	67.2
6170	o.g.	6.3	14.2	14.2	40.0
7287	o.g.	6.3	11.9	11.9	80.0
9788	o.g.	6.3	14.2	10.0	20.0
	GMT		13.4	11.9	40.0
3568	i.m.	6.3	23.8	23.8	56.6
6061	i.m.	6.3	28.3	23.8	91.9
	GMT		26.0	23.8	72.1

Table 3: The results of the blood samples from the skunks, including the control animals; the titre of the virus neutralizing antibodies are expressed in IU/mL and determined by RFFIT (n.d: not determined).

Animal	Construct	B0 (day 5)	B1 (day 28)	B2 (day 42)	B3 (day 57)
9892	SAD dIND1	0.04	0.03	0.03	0.15
9893*	SAD dIND1	0.09	0.06	0.09	n.d.
9895	SAD dIND1	0.11	0.06	0.06	0.11
9899	control	0.06	n.d	n.d	3.44
9897	control	0.05	n.d	n.d	4.31

* Animal developed severe dermatitis and was excluded for challenge.

4. Discussion

One of the major concerns with the distribution of ORVs for wildlife in the environment is the risk that nontarget species, including humans, will contact these vaccine viruses. The safety concerns associated with the first generation attenuated ORV led to the development of different recombinant vaccine vectors expressing the rabies virus glycoprotein; for example, vaccinia virus, human adenovirus type 5, pseudorabies virus, canine herpesvirus, and canine adenovirus type 2 constructs [19–24]. Some of these replication competent vectors are based on human pathogens and are therefore also not without risks, especially in view of immunocompromised persons. Unfortunately, replication-deficient constructs like an E1-deleted human adenovirus type-5 expressing the rabies virus glycoprotein did not induce detectable rabies VNA after oral administration [25]. The balance that must be attained is constructing a viral delivery system that is fully attenuated to render it safe and prevent replication and yet have sufficient viral characteristics that allow uptake into permissive cells and protein production to induce an immune response. A further important limitation of some of these recombinant constructs is the interference of preexisting immunity to the vector virus, severely compromising its efficacy [26–28]. Preexisting immunity to the vector virus would not be

important for highly attenuated rabies virus constructs. Therefore, several rabies virus constructs have been selected or developed and tested for their potential use as a rabies vaccine, including constructs with site-directed deletions in the phosphoprotein or its complete deletion [29–31]. For example, removal of Dynein Light Chain 8 binding site motif substantially reduced viral transcription and replication in the central nervous system [32]. Another strategy was to make the expression of the essential phosphoprotein dependent on translation and not transcription [33]. The SAD dIND1 construct uses a different approach which is aimed at inducing an improved innate immune response in vaccinated animals. The deletion introduced by site-directed mutagenesis interferes with the virus countermeasures to inhibit induction of IFN [34]. The enhanced antiviral host response was demonstrated in mice that were inoculated i.c. with SAD dIND1 or with the parental strain SAD L16. While all mice inoculated with SAD L16 succumbed to rabies, all SAD dIND1 inoculated animals survived [9]. An oral vaccine candidate must be safe and efficacious, preferably conferring life-long immunity, after the consumption of a single bait [35]. Although all foxes vaccinated with SAD dIND1 were fully protected against the severe rabies virus challenge, the VNA was lower than observed in foxes offered a bait containing $10^{6.3}$ FFU of the vaccine strain SAD B19 [36]. The geometric mean titre of 27 foxes was 43.5 and 33.9 IU/mL 60 and 110 days after the animals had consumed a SAD B19 vaccine bait, respectively. The difference in VNA titre is more notable considering that the administration of the SAD B19 vaccine was by bait consumption instead of by direct oral gavage for SAD dIND1. The lower VNA titres observed in the SAD dIND1 vaccinated animals could have been a result of the IFN Type 1 induced shift towards a Th1 immune response [7]. However, it is more probably the reduced viral growth and antigenic presentation of the rabies virus glycoprotein to antigen presenting cells. An increased induction of IFN by SAD dIND-infected cells would result in limited viral spread because noninfected neighbouring cells have been placed into an "antiviral" state by expression of antiviral IFN stimulated genes through the IFN-signalling pathway [10]. Rabies virus released from primary infected cells replicate inefficiently in such cells. Unfortunately, SAD dIND1 failed to elicit detectable levels of VNA in skunks, and consequently none of the animals induced a protective immune response against the challenge. The reduced ability of SAD dIND1 to induce rabies VNA compared to the SAD B19 vaccine strain was also shown in skunks. During a previous safety study with SAD B19, 3 of seven skunks receiving$10^{7.9}$ FFU by direct oral gavage seroconverted, and all three animals had measurable levels of rabies virus VNA (>5.0 IU/mL) 296 days after vaccination [37]. From this study, it can be concluded that the enhanced IFN production in response to SAD dIND1 results in a strong antiviral effect that outperforms the acknowledged immune-stimulatory effect of type I IFN. In order to make the highly attenuated replication competent virus, SAD dIND, an effective rabies vaccine candidate for oral vaccination of species including the striped skunk its immunogenicity must be improved. This could be achieved by altering the deleted domain, whereby the inhibitory effect on IRF-3 activation can be adjusted. For example, another construct (SAD dIND2) with a phosphoprotein lacking amino acids 182–186 instead of 176–181 (SAD dIND1) inhibits IRF-3 activation less efficiently [9]. Therefore, it can be assumed that, in SAD dIND2 infected hosts, there is less IFN Type 1 induction subsequently leading to more pronounced viral spread and antigenic presentation. However, SAD dIND2 was in contrast to SAD dIND1 not completely avirulent in mice [9], once more underscoring the difficulties in determining the delicate balance between safety and efficacy.

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