THE EFFECT OF INADEQUATE COLD CHAIN ON RABIES VACCINE POTENCY

A thesis submitted to Dr MGR Medical University, Chennai, in partial fulfillment of requirement for the degree of Doctor of Medicine in Pharmacology (Branch VI) Examination to be held in March 2007.

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This thesis is dedicated, with infinite gratitude,
to all my teachers, past and present.

Certificate

This is to certify that the dissertation entitled **" THE EFFECT OF INADEQUATE COLD CHAIN ON RABIES VACCINE POTENCY "** is the bonafide original work of Dr. Prasanna Kumar TS toward the MD - Branch VI (Pharmacology) Degree, Examination of the Tamil Nadu Dr. MGR Medical University, Chennai, to be conducted in March 2007.This study has not been submitted in full or in part to any other University.

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Declaration

I, Dr Prasanna Kumar TS, do hereby declare that this thesis entitled **“ THE EFFECT OF INADEQUATE COLD CHAIN ON PURIFIED CHICK EMBRYO RABIES VACCINE POTENCY "** has not been submitted by me for the award of degree, in part or whole, to any other University.

Dr Prasanna Kumar TS

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Introduction

Rabies is highly fatal viral encephalitis caused by a number of different strains of highly neurotrophic viruses [1] belonging to a single serotype in the genus lyssavirus, family rhabdoviridae. Immunization, both active and passive, remains the mainstay of prophylaxis against rabies. Cell culture based rabies vaccines are slowly replacing the nervous tissue rabies vaccine in prophylaxis against rabies [2]. However isolated reports of development of rabies, despite immunization with cell culture derived rabies vaccine, from developing countries including India, are questioning the authenticity of prophylactic measures and the vaccines used for prophylaxis [3, 4, 5, 6,7]. One of the proposed causes for cell culture derived rabies vaccine failure is being the inadequate vaccine potency due to exposure to non-ideal temperatures during storage and transportation [3,7]. In India, similar to other developing countries, due to frequent power cut, lack of storage facilities and trained staff, maintenance of cold chain for vaccines is often inadequate. Therefore estimation of the potency of cell culture rabies vaccines from the community has been suggested [3].

This prompted us to study the rabies vaccine storage practices in pharmacies based in three districts spread across three south Indian states and to estimate the potency of cell culture rabies vaccines obtained from the community.

Aim and Objectives

Aim

To determine whether reduced potency due to inadequate cold chain maintenance is the cause for cell culture derived rabies vaccine failure.

Objectives

1. To study the rabies vaccine storage practices in the community.
2. To estimate the glycoprotein content in cell culture derived rabies vaccines as an indirect measure of vaccine potency.

REVIEW OF LITERATURE

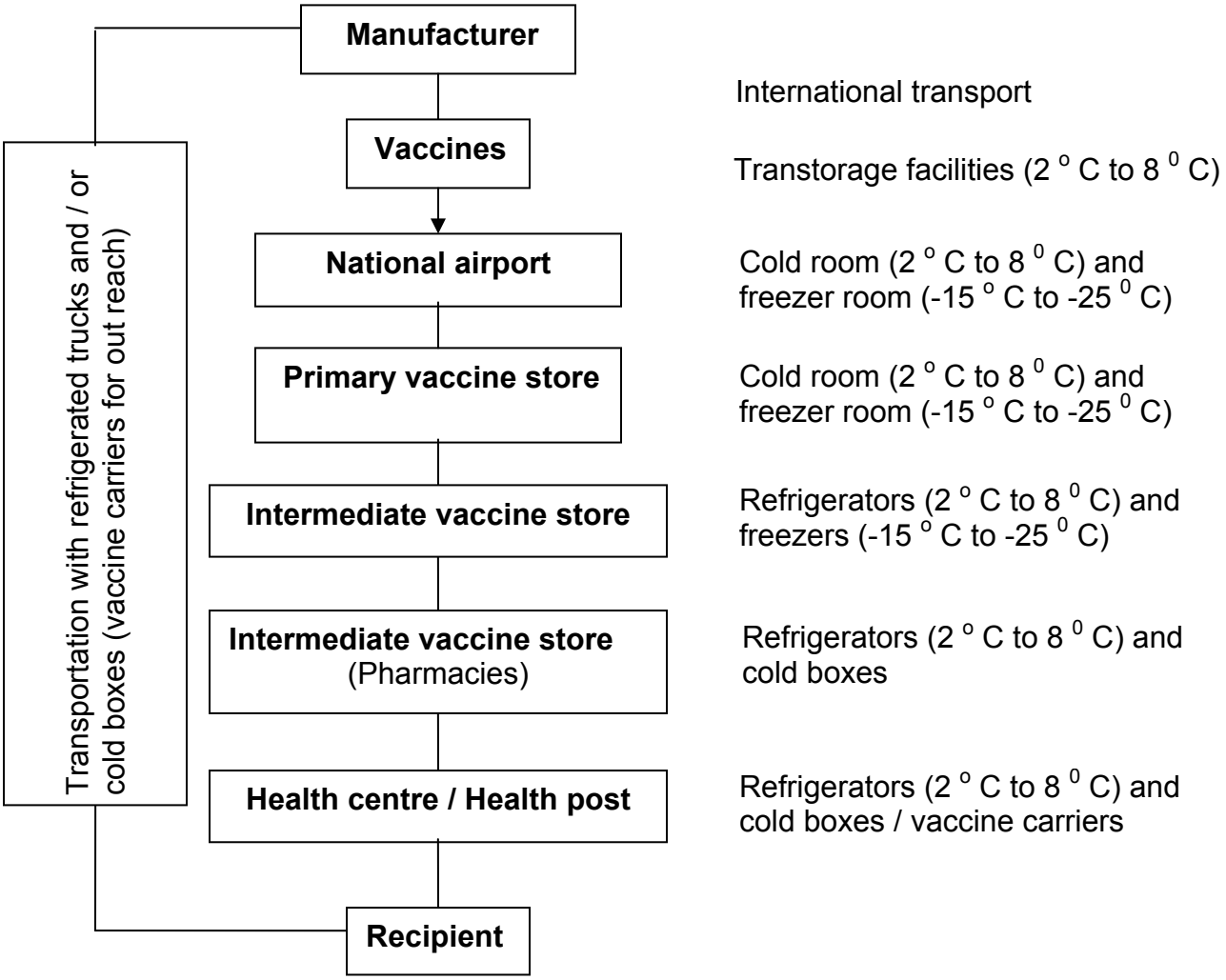
Cold Chain

Vaccines are biological products and are susceptible to fluctuations in temperature. Vaccines must be stored properly from the time they are manufactured until the time they are administered. Excessive heat or cold exposure damages vaccine, resulting in loss of potency [8]. Once potency is lost, it can never be restored. Furthermore, each time the vaccine is exposed to heat or cold, the loss of potency increases and eventually, if the cold chain is not correctly maintained, all potency will be lost, and the vaccine becomes useless. In some cases, heat exposure leading to loss of vaccine potency may also cause the vaccine to become more reactogenic [9]. The system used to keep and distribute vaccines in good condition is called the "cold chain." [9] This consists of a series of storage and transport links, all of which are designed to keep the vaccine at the correct temperature until it reaches the consumer. The cold chain has three main components: transport and storage equipment, trained personnel, and efficient management procedures. All three elements must combine to ensure safe vaccine transport and storage.

The cold chain begins with the refrigerator or freezer at the vaccine manufacturing plant, extends through the transfer of vaccine to the distributor and then to the provider's office, and ends with the administration of the

vaccine to the recipient [9]. Proper storage temperatures must be maintained at every link in the chain.

Fig 1. A typical cold chain system for vaccines



Requirements during vaccine transportation

Manufacturers and central pharmacies ship their vaccines in insulated containers, with documented ability to maintain the appropriate temperature for the anticipated maximum length of time required for the transportation, carrying appropriate number of ice packs. Containers should be fitted with both heat and cold monitors [10]. In case of long distance shipments they should use electronic monitors to detect possible problems and their location. Shipping boxes for most vaccines should be clearly labeled as containing perishable goods that have to be stored between 2 and 8^{o} C and must not be frozen. To avoid freezing, vaccines should not be placed directly on the ice pack [11]. If evidence of freezing is present when vaccines sensitive to freezing are received (e.g., results of temperature measurements), the vaccine must not be used [11].

Vaccine storage requirements

Vaccines should be stored in the refrigerator as soon as they are received. Vaccines should never be removed from the refrigerator except for the following reasons: withdrawing a dose(s); shipping to clients; or transporting to immunization clinics. The refrigerator door should not be opened too frequently. The World Health Organization recommends that the door should not be opened more than four times a day [12].

Any refrigerator or freezer used for vaccine storage must maintain the required temperature range year-round, be large enough to hold the year's largest inventory, and be dedicated to storage of biologics i.e., food or beverages should not be stored in vaccine storage units [13]. If there is an accumulation of more than 1 cm ice in the freezer compartment of a refrigerator, defrosting is required. Vaccines should be transferred to a vaccine carrier box or another refrigerator while this is being done [14]. The temperature should be monitored during this emergency period. Refrigerator plugs should be protected in an area where they cannot be knocked out accidentally. Procedures in the event of vaccine refrigerator failure should be posted on or near all such refrigerators. If a power outage occurs, all vaccines should be transferred into a thermal cold box or a container with ice packs, until the vaccines can be transferred to another refrigerator. If a short power outage is anticipated (less than 1 hour), the storage unit should not be opened. If a longer power outage is anticipated, then plans for transfer are warranted. Regular maintenance of refrigerators (cleaning coils, replacing door seals, etc.) should be performed and records kept.

Vaccines should never be stored on refrigerator door shelves because temperatures are warmer there than on the shelves of the refrigerator[13]. Space should be left between the products in the refrigerator to allow air to circulate. For freeze-dried products, for which diluent is provided in separate packages, the diluent should be stored at room

temperature to conserve refrigerator space, unless the vaccine direction insert specifies that the diluents must be refrigerated. All adsorbed vaccines should be kept away from the freezing element and away from direct contact with ice. Water bottles should be kept at the bottom, the top, and in the door spaces of the vaccine refrigerator, and ice packs should be kept in the freezer compartment to maintain a more constant temperature if there is a power failure. Educational material on the cold chain should be available in all centers storing vaccines.

All staff handling vaccines should have training about the importance of good vaccine storage, correct storage temperatures for the various vaccines and transportation techniques. They should know how to read and interpret maximum-minimum thermometers. One person should be identified as responsible for vaccine management. Another individual should also be trained for when the first person is absent.

Vaccine refrigerator should be placed in a room with a lockable door to prevent unauthorized handling or refrigerator entry after office hours. If this is not possible, a low traffic area should be considered. Refrigerators with lockable doors should be secured after office hours. If a product(s) is (are) known to have been exposed to temperatures outside of the recommended range, the exposed product(s) should be put in a box marked "DO NOT USE" and placed in a functioning refrigerator. The types

of products exposed should be recorded, as well as the duration and temperature of exposure, and advice on whether these products may be used or returned should be sought immediately.

Temperature monitoring

Proper temperature monitoring is key to proper cold chain management [15]. All vaccine storage refrigerators should have a maximum minimum thermometer or, if large quantities of vaccines stored, a continuous temperature recording device. Thermometers should be placed in a central location in the storage unit, adjacent to the vaccine. Different types of thermometers can be used, like standard fluid-filled, min-max, and continuous chart recorder thermometers [15]. Comparison of different thermometers used for monitoring vaccine storage compartment temperatures is given in Table 1. All monitoring devices should be certified or calibrated routinely. All refrigerators containing large quantities of vaccine (e. g central vaccine distributing areas) should also be connected to a temperature alarm monitoring system. Two daily temperature readings for the vaccine refrigerator should be taken and recorded — one in the morning when arriving and one at the end of the day — to ensure temperatures remain between 2 and 8^{o}C[15]. A chart-recording thermometer should also be checked for temperature fluctuations, which may occur between readings. In addition, temperature indicators (e.g., Freeze Watch™ [3M, St. Paul, Minnesota] or Cold Mark™ [Cold Ice, Inc., Oakland,

California]) can be considered as a backup monitoring system. However, such indicators should not be used as a substitute for twice daily temperature readings and documentation.

Table 1: Comparison of thermometers used to monitor vaccine temperatures

Thermometer type	Advantages	Disadvantages
Standard fluid filled	Inexpensive & simple	Less accurate (+ / - 1^0 C)
		No information on duration of out of specification exposure
		Cannot be recalibrated
Min – Max	Inexpensive	Less accurate (+ / - 1^0 C)
		No information on duration of out of specification exposure
		Cannot be recalibrated
Continuous recorder	Most accurate	Most expensive
	Continuous 24-hr readings of temperature range and duration	Requires training and maintenance
	Can be recalibrated at regular intervals	

The majority of commonly recommended vaccines require storage temperatures of 35 to 46°F (2 to 8°C) and must not be exposed to freezing temperatures.

Table 2: Vaccines and testing solution that must be stored between 2 and 8°C

1. Diphtheria and tetanus toxoids
2. Diphtheria and tetanus toxoids and pertusis vaccine (acellular)
3. Diphtheria and tetanus toxoids, adsorbed pertusis vaccine (acellular) and inactivated polio vaccine
4. Diphtheria and tetanus toxoids, adsorbed pertusis vaccine (acellular) , inactivated polio vaccine and haemophilus influenzae type b (Hib) conjugate vaccine
5. Hepatitis A vaccine
6. Hepatitis B vaccine
7. Hib conjugate vaccine
8. Inactivated polio vaccine
9. Influenza vaccine
10. Mumps, measles , rubella vaccine *
11. Meningococcal C vaccine
12. Pneumococcal 7- valent conjugate vaccine
13. Pneumococcal polysaccharide 23- valent vaccine
14. Rabies vaccine
15. Rabies immunoglobulin
16. Tuberculosis testing solution
17. Varicella vaccine *

* The diluents of these vaccines do not have to be stored in the refrigerator

Inadequacy in vaccine cold chain maintenance

Good practices to maintain proper vaccine storage and handling can ensure that the full benefit of immunization is realized. Provision of adequate equipment and training for staff in maintaining the "cold chain" and the use and care of equipment are important components of a successful immunization programme [16]. In recent years, instances of improper vaccine storage have been reported. An estimated 17 to 37% of providers expose vaccines to improper storage temperatures [17, 18]. Poor knowledge about storage and handling of vaccines by vaccine handlers, refrigerator temperatures outside the recommended range [19], using old and defective refrigerators for storing vaccines [20], frequent power cuts and lack of alternate power facilities [21] seem to be responsible for cold chain breakdown in the community based pharmacies. In India also, similar scenario exists as reports suggest that poor knowledge and improper practice of vaccine storage is common among the vaccine handlers [22,23] and defective power supply is affecting the cold chain [24] .

Rabies

Rabies is a zoonosis of certain mammalian species, endemic in all continents. Only a few European countries, some islands and peninsulas and Antarctica, are free of rabies, although imported infection is a universal risk. The urban enzootic in domestic dogs is of most importance to man, and is the cause of more than 95% of human rabies cases. The

incidence of rabies is unknown due to the gross under-reporting of an untreatable disease whose victims often choose to die at home, to avoid the expense of medical care. The highest recognized human mortality is in Asia. In India alone 30000 deaths (3 /10^5 population) were reported to WHO in 1998 [25]. Human rabies continues to be endemic in India except for the islands of Andaman, Nicobar, and Lakshadweep. According to a recent report the annual incidence of human rabies in India is estimated to be 20565 or about 2 per 100000 populations [26]. The majority of the victims in India are male, adult, from rural areas, and unvaccinated [26].

The lyssaviruses

Rabies is caused by the bullet-shaped RNA virus from *Rhabdoviridae* family. The ribonucleoprotein complex core of the virus is covered by a glycoprotein coat bearing projecting spikes [27]. Rabies is the first of seven genotypes. The other six genotypes are rabies-related viruses [28], five of which can cause fatal infection [29]. Two genotypes (5 and 6) are European bat lyssaviruses found in insectivorous bats across Europe. Australian bat lyssavirus (genotype 7) was discovered in flying foxes (fruit bats, genus *Pteropus*), in 1996 [30]. Two further genotypes (3 and 4), Mokola virus found in shrews and cats, and Duvenhage virus found in bats, are very occasionally seen in Africa [27,29]. The *Lyssavirus* genus has recently been divided into two phylogroups based on serological and genetic analyses [28]. Phylogroup I comprise all these

viruses except Mokola virus, which is in phylogroup II with Lagos Bat virus, which has not been found in humans. All phylogroup I genotypes have caused fatal rabies-like encephalitis in man, whereas Mokola virus has probably only caused three known human infections, one of which was a fatal encephalitis without any typical features of rabies [29]. Experimentally, phylogroup II viruses are less pathogenic, and there is little if any cross-neutralization with the phylogroup I lyssaviruses. This is of practical relevance for post-exposure treatment.

Transmission

Rabies is an infection initially of wild and then domestic animals, which is spread to humans by bites, contact with mucosal membranes, and (to a much lesser extent) aerosol inhalation in bat caves. Most infections (90%) are transmitted via domestic animals (cats and dogs), mainly due to their close association with humans [31]. Infection can occasionally occur via scratches infected with saliva, though the infection rate is 50 times lower [32]. The main animals responsible for bites are dogs (96.2%), most of which are stray. The most common bite sites are the extremities [26]. Human to human transmission has not been recorded, with the exception of six iatrogenic cases resulting from corneal graft implants [33] and four cases of organ transplant [34]. Airborne transmission is thought to have occurred in two men who inhaled virus aerosols generated in caves inhabited by rabid bats, and in a laboratory worker who became infected

while rabid sheep brains were being ground for vaccine production [35] .The virus may be shed in breast milk, and there has been at least one suspected case of transmission from a mother to her breastfed infant [36]. Transplacental infection occurs in animals [37] but has not been reported in humans. A number of women with rabies encephalitis are known to have delivered healthy babies.

Pathogenesis

After a bite, the virus replicates in muscle cells close to the site of the bite and then ascends to the central nervous system via the peripheral nerves [38]. On reaching the central nervous system, there is massive viral replication on membranes within neurons [39]. Later through retrograde axonal transport from the brain, via somatic and autonomic efferent nerves, virus is deposited in many tissues [39] including: skeletal and cardiac muscle, adrenal medulla, kidney, taste buds, respiratory tract, cornea, and nerve twiglets in the hair follicles. At this stage, productive viral replication occurs, with budding from outer cell membranes in the salivary, lacrimal and other glands, which permits further transmission of rabies by bites to other mammals.

The incubation period from bite to disease varies widely, but is usually between 30 and 90 days. Antigenic analysis has confirmed incubation periods of up to 7 years [40], although this is exceptional. Bites on the head and neck have a shorter incubation period (sometimes even as

short as 15 days) compared with those on the trunk and lower extremities, due to the decreased length and greater number of neurons. A recent report from India also suggests a disease incubation period ranging from two weeks to six months [26].

Following infection, the brain, spinal cord, and peripheral nerves show ganglion cell degeneration, perineural and perivascular mononuclear cell infiltration, and neuronophagia [41]. Inflammation is most marked in the midbrain and medulla in furious rabies [42] and in the spinal cord in paralytic rabies [43]. Vascular lesions such as thrombosis and hemorrhage are also described, mainly in the brainstem, hypothalamus, and limbic system. Outside the nervous system, there is focal degeneration of the salivary and lachrymal glands, pancreas, and adrenal medullae [44].

Immunology

Rabies virus evades recognition by the immune system until the late stage of the disease. At the site of inoculation, some virus may be briefly exposed, but once within neurons, virions and their antigens are hidden from immune surveillance. When virus is eventually secreted, rabies antigens are expressed on cell surfaces but the virus is then widely distributed throughout the body and an immune response is too late to combat the overwhelming infection. Neutralizing antibody usually appears during the second week of illness in unvaccinated patients, and remains at low levels until death a few days later [45]. Evidence of cell mediated

immunity, by lymphocyte transformation tests, was found in six of nine furious encephalitis patients, but not in seven with paralytic disease [46], who had few B cells. Both groups had reduced NK cell levels [47]. Studies of encephalitis in rodents show that survival or delayed mortality are associated with the early appearance of IFN-ı in the brain and neutralizing antibody [48,49,50] and also inflammation and up- regulation of MHC class II mRNA expression in CNS cells [51].

Rabies virus glycoprotein

The rabies virus glycoprotein is a major contributor to the pathogenicity of the virus [52]. Rabies virus glycoprotein consists of three domains: cytoplasmic, transmembrane and ectodomain. It occurs in a complete, membrane-bound form within infected cells, but it is released from them in a deleted, secreted form lacking the transmembrane domain [53]. Several rabies virus glycoprotein associated pathogenic mechanisms have been identified: (i) rabies virus glycoprotein must interact effectively with cell surface molecules that can mediate rapid virus uptake [54]. (ii) rabies virus glycoprotein is essential for efficient virus budding [55] (iii) expression levels of rabies virus glycoprotein must be controlled to prevent functional impairment of the infected neuron [56] and (iv) the transmembrane domain of rabies virus glycoprotein is important for generating protection against rabies and should be present in rabies virus DNA vaccines . The cell culture rabies vaccine potency depends on the amount of

immunogenic rabies viral glycoprotein antigen in the vaccine preparation[57]. Estimation of the rabies viral glycoprotein antigen, using a specific monoclonal antibody based ELISA [57] and single-radial - immunodiffusion (SRD) are being promoted as alternatives to the conventional in vivo tests for immunogenicity [58].

CLINICAL FEATURES

Prodromal symptoms

Itching or paraesthesiae at the site of the healed bite wound are the only specific prodromal symptoms, occurring in about 40% of patients. Non-specific features include: fever, headache, myalgia, fatigue, sore throat, gastrointestinal symptoms, irritability, anxiety and insomnia. Psychiatric disease may be suspected. The disease progresses to either furious or paralytic rabies encephalitis, usually within a week [59].

Furious rabies

This well-known form is recognized with characteristic hydrophobic spasms and sometimes with generalized extension, convulsions and opisthotonos posture [60]. Respiratory or cardiac arrest following a hydrophobic spasm is fatal in one-third of cases. Excitation, aggression, anxiety or hallucinations occur between calm, lucid intervals, when no neurological abnormality may be detectable. Other features include cardiac arrhythmias, myocarditis, labile blood pressure and temperature,

respiratory disturbances (e.g. Cheyne–Stokes respiration, cluster breathing), meningismus, III, VII and IX cranial nerve palsies, abnormal pupillary responses, muscle fasciculation, autonomic stimulation with lacrimation and salivation and rarely increased libido, priapism and spontaneous orgasms [60]. Coma eventually ensues, with flaccid paralysis, and the illness rarely lasts more than a week without intensive care.

Paralytic rabies

Less common than furious rabies, paralytic or 'dumb' rabies may be missed unless there is a high level of suspicion. Paralytic disease is characteristic of vampire bat transmitted rabies and is more common following infections by attenuated viruses, and perhaps after post-exposure vaccination [29]. Prodromal symptoms are followed by paraesthesiae or hypotonic weakness, commonly starting near the site of the bite and spreading cranially. Fasciculation, myoedema or piloerection may be seen. The ascending paralysis results in constipation, urinary retention, respiratory failure and inability to swallow. Flaccid paralysis, especially of proximal muscles, is associated with loss of tendon and plantar reflexes, but sensation is often normal. Hydrophobic spasms may occur in the terminal phase and death ensues after 1 to 3 weeks [60].

Management

In case of suspected animal bites, aim is to prevent the development of rabies. The most important steps are thorough cleaning of the wound and use of post-exposure prophylaxis, which together reduce the risk from a known rabid animal bite from 37 to 60% to nearly zero [61]. In case of large gaping wounds suturing should be postponed. Tetanus prophylaxis should be given. A prophylactic antimicrobial therapy should be given for serious bites or those on the hands. Once rabies symptoms have developed, treatment itself is mainly supportive, as the prognosis is poor. Patients must be heavily sedated in order to control their pain and terror. Ketamine has been suggested as an appropriate agent for this purpose [62]. The mainstay of treatment is intensive care support, including paralysis, sedation, and ventilation. Patients given intensive care develop complications such as cardiac arrhythmias, cardiac and respiratory failure, raised intracranial pressure, convulsions, fluid and electrolyte disturbances including diabetes insipidus and inappropriate secretion of antidiuretic hormone, and hyperpyrexia. Antiviral agents including intrathecal tribavirin (ribavirin) and interferon, rabies immunoglobulin, corticosteroids and other immunosuppressive drugs have proved useless in treating rabies [63].

Survival after rabies encephalitis

Although humans with paralytic rabies can survive for several weeks, especially with intensive care [64] the illness progresses persistently.

Till now only six patients have been documented as surviving rabies infection [65]. Survivors are usually left with neurological deficits [62].

RABIES PROPHYLAXIS

Active immunization - Rabies vaccines

Rabies vaccine preparations are fluid or dried preparations of rabies "fixed" virus grown in the neural tissues of rabbits, sheep, goats, mice or rats or in embryonated duck eggs, or in cell cultures, and inactivated by treatment with phenol or β-propiolactone . Five groups of rabies fixed strains are used to produce human rabies vaccines: Pasteur, Beijing, Flury, Fuenzalida and Street-Alabama-Dufferin (SAD) strains. The Pasteur-derived strains, Pasteur virus (PV) and Pitman-Moore (PM) strains), are most widely used for the production of traditional vaccines of the Semple or Suckling Mouse Brain (SMB) types, but also for the production of modern cell culture vaccines. The different rabies vaccines are classified according to the cell system used to cultivate the virus. Animal systems are still employed to produce the old traditional vaccines - Semple and SMB - which continue to be produced in several countries. Primary cell systems, particularly Hamster kidney and Chick embryo cells, are also used. Cell lines are presently the latest approach for vaccine production. Following Pasteur's initial development of rabies vaccine a variety of vaccines have been developed.

There are three types of rabies vaccines

1. Nervous tissue vaccines (NTV) –

    a. derived from adult animal nervous tissue (e.g. sheep)

    b. derived from suckling mouse brain

2. Duck embryo vaccine (DEV)

3. Cell-culture vaccines -

    a. Human diploid cell (HDC) vaccines

    b. “second generation” tissue culture (animal cell) vaccines

Three types of cell culture vaccines, one duck embryo vaccine and a nervous tissue vaccine are presently available in India [66].

Nervous tissue vaccines

A phenolized nerve-tissue vaccine was originally developed in India in 1911 by Sir David Semple at the central research institute, Kasauli , Himachal Pradesh.This is made from PV-11 strain of Pasteur fixed rabies virus infected sheep (Semple vaccine), goat, or mouse brains and is used in many parts of the world including Asia, Africa, and South America [67]. It has a low potency per dose, and a complete treatment involves up to 23 painful injections. NTVs are crude preparations capable of causing postvaccinal encephalitis with substantial frequency (0.05%).

The suckling-mouse brain (SMB) vaccine was developed at the Bacteriological Institute of Chile in 1954. This vaccine was originally used in dogs; use in humans was begun experimentally in 1960. Suckling mouse brain vaccine (SMB vaccine) is an improvement over adult animal NTVs as they have been prepared from the brains of suckling mice (less than 9 days old) which lack or contain less amount of myelin in their nervous tissue. Three strains of fixed rabies virus are used for the preparation of this vaccine: 2 strains isolated in Chile (strains 51 and 91, of dog and human origin respectively) and the challenge virus standard (CVS) strain. SMB vaccine is given in 10 daily doses plus booster doses 10 and 20 days after the end of the primary series. These are extensively used in most of the Caribbean and all countries of Latin America. Its immunogenic potency is determined by the National Institutes of Health (NIH) test.

Purified duck embryo vaccine (PDEV)

PDEV has been developed in an attempt to eliminate neuroparalytic factors contained in the nervous tissue vaccines. The rabies virus is grown in embryonated duck eggs. Incidence of neuroparalytic reactions are less, however anaphylactic reactions are frequent. The antigenecity is low so that many (16 to 25) doses have to be given to obtain a satisfactory post exposure antibody response. It is available as “Vaxirab” (Zydus Recon) in India.

Purified chick embryo cell vaccine (PCECV)

This vaccine is prepared from the fixed rabies virus strain Flury LEP grown in chicken fibroblasts. It is inactivated with β-propiolactone and further purified by zonal centrifugation. The vaccine potency is determined by the NIH test and the modified antibody-binding tests [68]. The vaccine may be used up to 48 months from the date of release. It should be kept between 2 and 8°C [3]. Rabipur ® is a highly purified, potent and efficacious PCECV rabies vaccine. It is the most widely used modern cell-culture rabies vaccine . In India also PCECV is marketed as “Rabipur” (Aventis Pastuer).

Purified vero cell vaccine (PVCV)

The Vero cell line was established in 1962, starting from a primary culture of vervet monkey (Cercopithecus aethiops) kidney cells. The heteroploid VERO cell line was introduced in 1982 to the production of inactivated rabies vaccine; it has all the advantages of the Human Diploid Cell system. The Pitman-Moore (PM) strain of fixed rabies virus is used, after adaptation to growth in WI-38 cells. The virus suspension is inactivated using β-propiolactone. The vaccine may be used up to 36 months from the date of release. It should be kept between 2 and 8°C after release [3]. In India two brands of PVRV, “verorab” (Ranbaxy) and “Abhyarab” (Indian Immunologicals) are available.

Rabies vaccine, adsorbed (RVA)

This vaccine is made in a diploid cell line derived from fetal rhesus monkey lung cells. The vaccine virus is inactivated with β-propiolactone and concentrated by adsorption to aluminium phosphate. Currently it is not available in India.

Human diploid cell vaccine (HDCV)

The use of the human diploid cell system permitted the development of the HDCV, one of the most widely distributed cell culture rabies vaccine, and today considered as the reference vaccine. An inactivated rabies vaccine for human use was first prepared in cell culture in 1964. In 1966 it was shown that the human diploid cell (HDC) strain WI-38 was a suitable substrate for the propagation of the Pitman -Moore (PM) stain of fixed rabies virus. The vaccine was first licensed for use in France in 1974 and commercial production started in 1978. The virus is now cultivated in MRC-5 human diploid cells, which are propagated and controlled according to the recommendations published by WHO and the regulations of national authorities. The vaccine virus is inactivated with β-propiolactone and concentrated by ultrafiltration. No serious anaphylactic or encephalitic reactions have been reported. The potency of each lot of vaccine is determined by the NIH-Test [68]. In India HDCV “Rabivax” is manufactured by the Serum Instute of India.

Rabies vaccine potency testing

NIH test

The potency of the rabies vaccine is established by the NIH test [68]. Groups of at least ten mice, aged 3 to 4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia [69], or with two doses, 1-week apart, according to the NIH test [70]. A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge with fixed-strain rabies virus. Many criticisms have been made regarding the NIH test in particular the high variability of the results, the fact that the test does not reproduce natural conditions of rabies vaccine use and that the choice of challenge strain affects NIH values [57]. NIH test also induces substantial distress and suffering in laboratory mice. Since it involves use of virulent rabies virus it raises safety concerns and requires stringent biosafety measures.

Immunocapture methods

The vaccine potency depends on the amount of immunogenic rabies viral glycoprotein antigen in the vaccine preparation [57]. The immunocapture methods based on the use of a neutralizing monoclonal anti-glycoprotein antibody seems to be convenient tools for the determination of the in vitro potency of rabies vaccine [71]. The glycoprotein content of rabies vaccines containing the Pitman-Moore strain of rabies

virus measured by the single radial immunodiffusion assay has been shown to be correlating well with vaccine potency [72]. An ELISA test using a specific monoclonal antibody to quantify the immunogenic rabies viral glycoprotein antigen in the inactivated rabies viral antigen preparation also indicated a good correlation with the NIH mouse potency assay [57]. These two in vitro tests, i.e. single radial immunodiffusion test and ELISA, have been recommended by the WHO, for the partial replacement of in vivo potency test [73].

Adverse effects of tissue culture vaccines

Mild erythema, pain, headache, malaise, fever occur and very rarely neurological illness has been reported. There is very wide variation in the incidence of side-effects. A systemic allergic reaction has been observed in 6% of those vaccinated in the USA, 3 to 13 days following late booster injections of HDCV [74]. The urticarial rash, angioedema and arthralgia respond to symptomatic therapy. It is possibly caused by an IgE mediated reaction to beta – propiolactone modified vaccine components [75].

Pre-exposure vaccine regimens

Pre-exposure prophylaxis regimen is given in Table 3. An economical intra dermal (i.d.) post-exposure treatment has been used for 15 years in Asia [76]. A booster dose by either route one year later enhances and prolongs the immune response, which lasts more than 10

years in 96% of people (intra muscular vaccine was used in this study)[77]. The level of antibody may be lower following i.d. vaccine, but the quality of the secondary immune response to a booster dose is similar for i.d and intra muscular (i.m.) injections. A booster dose or an antibody test should be performed six monthly for rabies laboratory staff and all those at continued high risk of infection. For other people, if the antirabies antibody titre is < 0.5 IU, booster doses should be given every three years, and if > 0.5 IU, boosters can be given every 10 years[78]. If no serology is available, or affordable, boost every 5 to 10 years. Measurement of neutralizing antibody levels after treatment is necessary only if immunosuppresion is suspected, or to determine whether a repeated booster dose is needed. Chloroquine antimalarial chemoprophylaxis may inhibit the induction of rabies antibody after i.d. vaccination[79], so the larger dose must be given i.m.

Post-exposure prophylaxis

Regimen for post exposure prophylaxis is given in Table **5**.

Table 3. Pre-exposure prophylaxis regimen

Risk category	Nature of risk	Typical populations	Pre-exposure recommendations
Continuous	Virus present continuously, often in high concentrations. Aerosol, mucous membrane, bite or non-bite exposure. Specific exposures may go unrecognized.	Rabies research lab worker [1], rabies biologics production workers.	Primary course. Serologic testing every 6 months; booster vaccination when antibody level falls below acceptable level [2]
Frequent	Exposure usually episodic, with source recognized, but exposure may also be unrecognized. Aerosol, mucus membrane, bite, or non-bite exposure.	Rabies diagnostic lab workers [1], spelunkers, veterinarians and staff, and animal-control and wildlife workers in rabies endemic areas. Travelers visiting foreign areas of endemic rabies for more than 30 days.	Primary course. Serologic testing or booster vaccination every 2 years [2]
Infrequent (greater than population at large)	Exposure nearly always episodic with source recognized. Mucous membrane, bite, or non-bite exposure	Veterinarians and animal control and wildlife workers in areas of low rabies endemnicity. veterinary students.	Primary course; no serologic testing or booster vaccination.
Rare (population at large)	Exposures always episodic. Mucous membrane, or bite with source unrecognized.	U.S. population at large, including persons in rabies endemic areas.	No vaccination necessary.

1 Judgment of relative risk and extra monitoring of vaccination status of laboratory workers is the responsibility of the laboratory supervisor.
2 Minimum acceptable antibody level is complete virus neutralization at a 1:5 serum dilution by RFFIT. Booster dose should be administered if the titer falls below this level.

Table 4. Rabies pre-exposure prophylaxis schedule

Type of Vaccination	**Route**	**Regimen**
Primary	IM	HDCV, RVA* , PCEC, 1.0 ml (deltoid area), On days 0, 7, and 21 or 28
Booster[1]	IM	HDCV, RVA*, PCEC, 1.0 ml (deltoid area), day 0 only

[1] Administration of routine booster dose of vaccine depends on exposure risk category as noted in the table above.

*Although this vaccine is licensed, it is currently unavailable.

Table 5. Regimen for Rabies Post-exposure Prophylaxis by vaccination Status

Vaccination Status of exposed person	**Treatment**	**Dosage / administration guidelines for all ages**	**Day of regimen**
Never previously vaccinated	Local wound cleansing Tetanus toxoid booster, if needed		
	Human Rabies Immune Globulin (HRIG)	20 IU/kg body weight, given once IM. As much as possible of the HRIG should be infiltrated into and around the wound, and the remaining given IM at a site distant from the vaccine (frequently the gluteals).	Day 0
	Rabies Vaccine	Five 1-ml doses, given IM. Rabies vaccine must be given in the deltoid area of adults and older children. The vaccine may be given in the anterolateral thigh of young children. Never give rabies vaccine in the gluteals.	Days 0, 3, 7, 14, 28
Previously vaccinated (Those who have completed a preexposure or postexposure regimen of Human Diploid Cell Vaccine (HDCV) or Purified Chick Embryo Cell Vaccine (PCEC), or who have received another vaccine and had a documented rabies antibody titer of > 1:5 by the Rapid Fluorescent Focus Inhibition Test (RFFIT)	Local wound cleansing Tetanus toxoid booster, if needed		
	Rabies Vaccine	Two 1- ml doses, given IM. Rabies vaccine must be given in the deltoid area. Never give rabies vaccine in the gluteals.	Days 0, 3

Passive immunization

Rabies immune globulin, human (HRIG)

HRIG is a gamma globulin prepared by cold ethanol fractionation from the plasma of hyperimmunized humans. There are fewer adverse reactions to HRIG than to equine antirabies serum. Serum sickness has not been reported with human RIG. HRIG is very expensive [80] and as such is not widely used in the third world countries that carry the highest risk rabid animal injuries.

Antirabies serum, equine

This is concentrated serum from horses' hyperimmunized with rabies virus. It is economical in comparison with HRIG however it carries risk of severe anaphylactic reactions on administration [81]. It has been used in countries where HRIG is not available.

Passive immunization regimens

Rabies immune globulin (RIG) should be given with every primary post-exposure treatment [76]. It is essential for severe exposure to infection: bites on the head, neck or hands or multiple bites. The WHO recommends that a skin test must be performed prior to the administration of ERIG in order to detect potential IgE- mediated hypersensitivity to equine serum proteins. A positive skin test does not rule out the use of ERIG when there is no alternative but special precautions should be taken to minimize

reactivity. Serum sickness is also a potential adverse reaction to ERIG and has been reported to occur 7 to 10 days after injection in approximately 1 to 6% of patients**.** Passive immunization provides some protection for the 7 to 10 days before vaccine induced immunity appears. RIG apparently neutralizes virus in the wound and enhances the T lymphocyte response to rabies [82]. The dose of 20 units / kg body weight of HRIG (or 40 units / kg equine RIG) should be infiltrated deep around the wound. If this is anatomically impossible, the rest may be given by i.m. injection at a site remote from the vaccine, but not into the gluteal region [83]. For multiple bites the RIG can be diluted two- or three-fold in saline to ensure infiltration of all wounds. The recommended dose of RIG must not be exceeded as this will impair the immune response to the vaccine.

Efficacy of post-exposure treatment

Indian studies have shown that the untreated mortality from proven rabid dog bites is 35 to 57% [29]. Optimal modern post-exposure treatment given on the day of the bite to healthy recipients is practically 100% effective. However recently John and Patnaik [6], reported development of rabies in an otherwise healthy 5 year old girl despite post-exposure prophylaxis with PCECV and HRIG. Similar isolated cases of development of rabies, despite complete and prompt treatment with modern products, have been reported in animals [84] and human beings i.e from India [6], Pakistan [7], Thailand [4,5] and sub-Saharan Africa [85]. These reports are

questioning the authenticity of cell culture rabies vaccines available in the community. One of the suggested causes for cell culture rabies vaccine failure is reduced potency of these vaccines due inadequate cold chain. Reports point towards the frequent power failures and the lack of refrigeration facilities in the community leading to loss of vaccine potency [7]. Hence a pilot study to evaluate the potency of rabies vaccines available in the community, i.e out of the hospital / laboratory, offered for prophylactic use in human and veterinary establishments has been indicated [3].

Materials and methods

A cross sectional survey to assess the adequacy of cold chain for cell culture rabies vaccines during transportation and storage.

In this cross sectional survey three districts of South India i.e. Vellore (Tamil Nadu), Kolar (Karnataka), and Chittoor (Andhra Pradesh) were chosen as study areas. A questionnaire was prepared keeping in mind the cold chain requirements for vaccine transport and storage (see annexure 1). A total of 102 pharmacy shops situated in the study areas, Vellore and Ranipet [Vellore Dist], Kolar and Mulbagal [Kolar Dist], Chittoor and Bangaarupalyam [Chittoor Dist], were contacted during the study. Of the 102 pharmacy shops contacted, 29 declined to participate and the rest seventy three pharmacy shops agreed to provide information on their vaccine storage system and allowed us to document the temperature in their vaccine storage compartments. A single observer visited the pharmacy shops and interviewed pharmacists using the earlier mentioned questionnaire. The type of refrigerator / freezer unit, temperature-monitoring equipment and records were noted, as were the locations of vaccines in refrigerator and freezer, and the presence of expired vaccines. Other information collected included the following: staff training, use of written guidelines, receipt of vaccine deliveries, different brands of cell culture rabies vaccines, number of vaccines being sold per month, average duration of rabies vaccine storage in the pharmacy and the professional educational level of the individuals designated as vaccine

handlers. At each pharmacy, interviewer measured the central core temperature of the vaccine refrigerator using standard fluid filled thermometer and noted the position of the vaccines in the refrigerator as well as the presence of other items.

Collection of cell culture rabies vaccines (PCECV) from the community based pharmacies

Based on the cross sectional survey, well maintained and poorly maintained pharmacies were identified. Eight PCECV vaccine vials were collected from eight poorly maintained pharmacies and eight PCECV vaccine vials were collected from eight well maintained pharmacies.

Heat simulation studies

Twenty vials of PCECV rabies vaccine from the same manufacturer's batch were collected from Christian Medical College Hospital Pharmacy. Christian Medical College is a tertiary care teaching hospital in South India. To simulate the exposure to high temperatures during storage, ten vials were incubated at 37 ^{0} C for 12 hours / day for 15 days. At the end of every incubation period vials were transferred to recommended storage conditions (2 to 8°C). The other ten PCECV vials were stored at recommended storage conditions (2 to 8°C) throughout the study.

Estimation of glycoprotein content in PCECV samples

Virus bound glycoprotein in the PCECV samples, collected from the community based pharmacies as well as vaccine vials used for heat simulation studies, was assessed using an enzyme linked immuno sorbent assay (ELISA) procedure described by **Fournier – Caruana et al, 2003** [86]**.** The monoclonal antibodies (mAb-D1), secondary antibodies, reference vaccine and experimental protocols were obtained from Institut Pasteur, Paris, France.

In-vitro test for rabies vaccine potency testing

Principle of the rabies glycoprotein enzyme immunoassay

ELISA represents one of the *in vitro* tests which can be used for rabies vaccine potency testing [86]. The principle of this test is based on the measurement of the glycoprotein content in rabies vaccine by ELISA [87] after the immunocapture of rabies virus glycoprotein [88]. The vaccine to be tested is incubated in a plate previously sensitized with anti-glycoprotein antibody (polyclonal or monoclonal antibody). Bound antigens are subsequently identified by adding the same antibody labelled with peroxidase which is revealed in the presence of substrate and chromogen. Comparison of absorbance measured for the tested vaccine with the absorbance of the reference vaccine permits the determination of the glycoprotein content.

Biological material

Monoclonal antibody for coating

Semi-purified Immunoglobulins (IgG) anti-rabies glycoprotein: purified from mouse ascitic fluid immunized with hybridome 01-25WI-1805 (Collection Nationale de Culture de Microorganismes-Institut Pasteur Paris). Specificity: anti-rabies glycoprotein (PV strain) direct to antigenic site III. IgG 01 A: 530 µl was diluted *1 / 200*

Peroxidase conjugate

The same IgGs conjugated with peroxidase.

IgG 01 A-PO: 530 µl was diluted *1/ 200*

Antigen for validation

Inactivated and purified rabies vaccine (PV strain). First dilution: *1/10* after hydration with 1 ml of distilled water. Antigen concentration after 1/10 dilution: 1000 ng / ml.

Preparation of monoclonal antibodies

In brief monoclonal antibodies were prepared as described by Lafon [89] after immunization of BALB/c mice with inactivated and purified rabies virus (Pasteur virus strain). MAb-D1 (IgG1 isotype) recognizes the antigenic site III (involved in virus neutralizing Ab induction) of native but not β-mercapto-ethanol and / or sodium dodecyl sulfate (SDS) - treated glycoprotein [90]. It neutralizes lyssaviruses belonging to the genotype 1 (rabies viruses: Pasteur virus (PV), challenge virus standard (CVS) and Pitman-Moore (PM) strains) and to the genotype 6 (European bat

lyssavirus 2 (EBL2)). Produced as ascitic fluid and diluted 1:10^6, it neutralizes 2 X 10^5 fluorescent focus units / ml of virus (PV strain), indicating that it possesses a very high virus neutralizing activity. Before its use in ELISA, the ascitic fluid was precipitated with ammonium sulfate (40% saturation) and centrifuged. The pellet was suspended in chilled distilled water and dialyzed against PBS. Concentrated immunoglobulins were sterilized by filtration and used to coat ELISA microplates and to prepare peroxidase conjugate as previously described [91].

Buffers and reagents

Carbonate 0.05 M

$NaCO_3$, 10 H_2O. ....14. 30 g

Distilled water.....to make 1000.00 m1

Bicarbonate 0.05M:

$NaHCO_3$ .....4.20 g

Distilled water.....to make 1000.00 m1

Carbonate buffer 0.05 M pH=9.6:

Bicarbonate 0.05 M..... 900.00 ml

Adjusted pH=9.6 with carbonate 0.05 M

Phosphate buffered saline pH=7 concentrated 10 times (PBS l0X):

NaCl..... 80.00 g KCl .....2 .00 g

Na_2PO_4, 12 H_2 0 11.33g KH_2PO_4……..2.OO g

Distilled water……to make 1 000.00 ml

PBS-Tween pH=7:

PBS 10 X 100.0 m1

Tween 20 .....0.5 m1

Distilled water..... to make 1000.0 ml

PBS-Tween-BSA pH=7:

PBS l0 X..... 10 .00 ml

Tween 20. ..... 0.05 ml

Bovine serum albumin (Fraction V).....0.50 g

Distilled water.....to make 100.00 ml

Adjusted pH= 7 with 4N NaOH

Buffer for peroxidase substrate pH=5.6 (citrate buffer):

Tri-sodium citrate, $2H_2O$ ($Na_3C_6H_5O_7$, $2H_2O$)..... 11.67 g

Citric acid, 1 H_2O ..... 2.l 7 g

Hydrogen peroxide 30% (110 vol)..... 1.00 m1

Distilled water..... to make1000.00 m1

Substrate-Chromogen solution:

Ortho - phenylene diamine..... 50 mg

Citrate buffer ..... 25 ml

Stopping solution: 4N sulfuric acid

Dilution id carried out in an ice bath

Cooled distilled water..... 80.00 m1

H_2SO_4, 36N.....10.00 ml

Safety measures

The washings were carefully carried out with an automatic apparatus (Fig 2b) and drying plate after inverting it on an absorbent paper after each washing. All reagents were adjusted to the laboratory temperature by waiting 10 min before use. Vaccines or infected cell supernatants to be tested are often inactivated nevertheless, samples were considered infectious and health and safety precautions were observed. Before working, the plan for distribution and identification of samples was established. Reference antigen or vaccine and samples were diluted in tubes and not in the sensitized plate.

Microplate sensitization

Microplate was sensitized with purified anti-glycoprotein monoclonal antibodies by adding in each well 200 µl of appropriate dilution of antibodies. [Different dilutions of antibody were previously tested to determine the optimal concentration. About 1 µg / well is generally required]. For sensitization, antibodies were diluted in carbonate buffer pH=9.6. After an incubation for 3 hour at 37^0 C in a humidified atmosphere (or covered with a sealer sheet) (Fig 2a), well content was aspirated and plate was inverted and dried on absorbent paper at laboratory temperature for 5 mn. Each well was then filled with 300 µl of 0.3% bovine serum albumin and 5% sucrose dissolved in carbonate buffer. After incubation at 37^0 C for 30 min, well content was

aspirated. The plate was inverted, dried for 1 min on absorbent paper at laboratory temperature and was immediately used or stored sealed at -20^0 C until use.

The assay

The sensitized plate was washed 5 times in PBS- Tween, inverted and dried for 1 min on absorbent paper. The first well (IA) (or all wells of the line 1) received 200 µl of PBS -Tween-BSA and served as blank control. Two hundred microliters of 5, two folds serial dilutions (in PBS – Tween -BSA) of the reference vaccine were distributed in duplicate in wells of the lines 2 and 3. The first dilution contains about 1 µg / ml of rabies glycoprotein. Two hundred µl of vaccines samples (serially two folds diluted in PBS-Tween-BSA) were distributed in duplicate in each well. The plate was covered with a sealer sheet and incubated for 1 hour at 37^0 C. Then, well content was aspirated and plate was washed 5 times with PBS - Tween. Two hundred µl of an appropriated dilution (in PBS –Tween -BSA) of peroxidase-labelled antibodies was distributed in all wells. After incubation for 1 hour at 37^0 C, labelled antibodies were aspirated and plate was washed 6 times with PBS -Tween, inverted and dried for 1 min on absorbent paper. Then, all wells received 200 µl of substrate -chromogen solution. Plate was sealed and incubated in dark at room temperature for 30 mn. A yellow-orange color was developed and the reaction was stopped by adding in each well 50 µl of stopping solution (Fig 2d).

Interpretation

The bottom of the plate was carefully wiped with absorbent paper. The optical density (OD) of each well was measured at 492 nm with an ELISA plate reader (Fig 2c). For the evaluation of glycoprotein content a reference vaccine was used. The reference vaccine contains purified viral particles with a well known glycoprotein content (µg / ml) directly determined (for example, determination of total viral protein and percentage of glycoprotein by SDS - polyacrylamide gel electrophoresis) or indirectly determined in ELISA using a calibrated vaccine. Standard curve was generated using spline parameter. The KC 4 software (version # 2.7,Rev # 8, Bio -Tek Instruments) was used to express the OD values as glycoprotein content in ng / ml, as previously used by other workers [92].

Sample size determination and statistical analysis

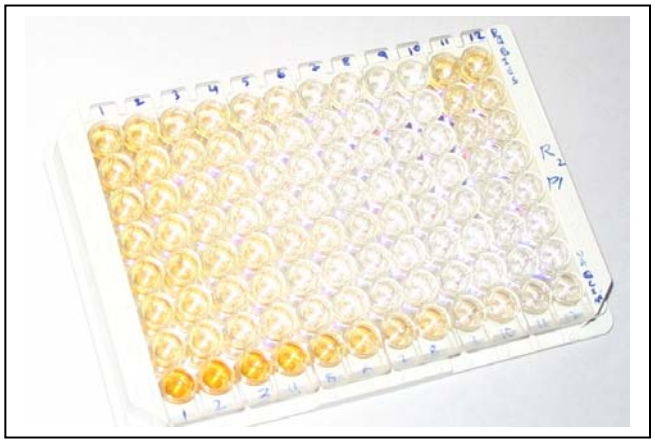
Sample size calculations of PCECV vials for the ELISA suggested large, unfeasible numbers. Hence, we chose the above stated numbers of PCECV vials and planned to do retrospective power analysis to determine whether our study suggests equivalence between the groups. Statistical analysis later suggested that it is not possible to show significant differences even with large sample sizes. The non-parametric test, Mann - Whitney U Test, was used for statistical comparison of the glycoprotein contents of the vaccines.

Fig 2.

2a. ELISA plate incubator

2b. Automated ELISA plate washer

2c. ELISA plate reader



2d. ELISA plate loaded with PCECV samples

Results

We contacted 102 pharmacies during the study, of which 73 pharmacies agreed to participate in the study. All these pharmacies were storing the PCECV “Rabipur”, while only 12 (16.4%) of these 73 pharmacies were storing the PVRV “Verorab”. Other brands of rabies vaccines like PDEV (Vaxirab), PVRV (Abhyarab) and HDCV (Rabivax) were not available in any of these pharmacies. “Rabipur” was the most commonly prescribed rabies vaccine followed by “Verorab”. None of these pharmacies were storing either equine antirabies serum or human rabies immunoglobulins. Pharmacies were obtaining these vaccines from Chennai (Vellore & Ranipet), Bangalore (Kolar & Mulbagal) and Thirupathi (Chittoor & Bangarupalaym). Average number of rabies vaccines sold / pharmacy / month was 3 - 5 in urban areas i.e Vellore, Kolar and Chittoor while the same was about 1-2 in semi urban areas i.e , Ranipet, Mulbagal and Bangarupalyam. The maximum duration of “in pharmacy storage” for a vaccine vial was about 6 months in all the pharmacies. Even though three different state electricity boards were supplying the electricity to these pharmacies, the pattern and duration of power outage was almost similar in all the pharmacies. Other results of the survey are given in Table 6. Our results show that rabies vaccines are exposed to non-ideal temperatures during transport and storage.

To assess the effect of poor cold chain on the potency of rabies vaccines we collected PCECV vials from poorly maintained (n=8) and well maintained pharmacies (n=8). We evaluated the potency of these vaccines indirectly by measuring the virus bound glycoprotein content in these vaccine samples. Our results (Tables 7, 8, 11, 12 & 15) show that there is no significant difference in the glycoprotein content of the vaccines collected from either poorly maintained and well maintained pharmacies.

The next experiment was conducted to evaluate the effect of high temperatures on the rabies vaccine potency. The rabies vaccines in the community are exposed to temperatures around 33 to 37^0C (usual temperature in these parts of South India) for a maximum about 180 hrs (average 1hour / day for of six months). Hence we simulated this condition in our laboratory by incubating the vaccines (n=10) at 37^0C for 180 hrs. Similar number (n=10) of vaccines from the same manufacturer's batch, stored at recommended temperature (2 to 8^0 C), were used as controls. Glycoprotein content was measured in all these vaccines as an indirect measure for vaccine potency. Our results (Tables 9,10,13,14 & 16) show that there is no significant difference in the glycoprotein content of these two groups of vaccines.

Table 6. Rabies vaccine storage practices in South India

Attribute	Total	Percentage
Number of Personnel Handling the Vaccines	289	
1. Trained	108	37.4
2. Untrained	181	62.6
Power Cuts (Duration / Day)		
1. 30 min – 1 hour	51	69.9
2. 1hr – 2 hrs	22	30.1
Alternate Power Facilities		
1. Available	11	15
2. Unavailable	62	85
Condition of the Refrigerator		
1. Satisfactory	27	37
2. Not satisfactory	46	63
Thermometer in the Vaccine Compartment		
1. Present	Nil	0
2. Absent	73	100
Recording of Vaccine Compartment Temperature		
1. Once daily	Nil	0
2. Once weekly	Nil	0
3. Once monthly	Nil	0
4. Occasionally	5	6.8
5. Never	68	93.2
Refrigerator Use		
1. Switched off at night	12	16.4
2. Used round the clock	61	83.6
Temperatures Documented During Interview*		
1. Within ideal range (2 - 8° C)		
2. Not within the ideal range	14	19.2
* minimum recorded temperature : 6° C	59	80.8
maximum recorded temperature : 32° C		
Maintenance of Cold Chain During Transport		
1. Adequate	48	65.8
2. Inadequate	25	34.2

Table 7. Vaccines from poorly maintained pharmacy shops - Mean OD values

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	**1.782**	**1.2875**	**0.8010**	**0.3895**	**0.1665**
1	0.5120	0.3075	0.1660	0.0800	0.0350
2	0.5075	0.2815	0.1280	0.0525	0.0190
3	0.4695	0.2645	0.0940	0.0320	0.0170
4	0.4375	0.2085	0.0760	0.0300	0.0150
5	0.5390	0.3190	0.1715	0.0840	0.0375
6	0.4715	0.2595	0.0855	0.0345	0.0190
7	0.4580	0.2690	0.1385	0.0640	0.0300
8	0.4625	0.2250	0.0825	0.0270	0.0295

Table 8. Vaccines from well maintained pharmacy shops – Mean OD values

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	1.3925	1.1965	0.6675	0.2930	0.1825
1	0.4570	0.2515	0.1400	0.0725	0.0250
2	0.4065	0.2315	0.0900	0.0435	0.0235
3	0.4030	0.2123	0.0890	0.0365	0.0245
4	0.3810	0.2375	0.0820	0.0365	0.0150
5	0.4385	0.2495	0.1295	0.0625	0.0330
6	0.4480	0.2390	0.0900	0.0565	0.0235
7	0.4395	0.2505	0.1225	0.0520	0.0265
Std	**1.0495**	**XXX**	**0.3930**	**0.2775**	**0.1440**
8	0.1715	0.1530	0.0425	0.0190	0.0130

Table 9. Non-heated vaccines - Mean OD values

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	**1.481**	**1.126**	**0.8695**	**0.284**	**0.210**
1	0.4065	0.1770	0.0785	0.0155	
2	0.3560	0.1455	0.0680		
3	0.2440	0.1455	0.0815		
4	0.2305	0.0880	0.0415		
5	0.4075	0.2090	0.0910	0.0155	
6	0.3120	0.1105	0.0680		
7	0.3790	0.2450	0.0810	0.0150	
Std	**1.0495**	**XXX**	**0.3930**	**0.2775**	**0.1180**
8	0.2565	0.1520	0.0785	0.0205	0.0165
9	0.2850	0.1600	0.0795	0.0220	0.0190
10	0.2635	0.1375	0.0685	0.0225	0.0120

Table 10. Heated Vaccines – Mean OD values

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	**0.8715**	**0.5940**	**0.4180**	**0.2280**	**0.0995**
1	0.3800	0.1915	0.1010	0.0380	0.0205
2	0.3185	0.1455	0.0670	0.0310	0.0121
3	0.2266	0.1340	0.0520	0.0250	0.0110
4	0.2630	0.1340	0.0580	0.0150	0.0130
5	0.3115	0.1715	0.0900	0.0390	0.0185
6	0.2280	0.1130	0.0610	0.0280	0.0140
7	0.3030	0.1545	0.0750	0.0285	0.0130
8	0.2790	0.1310	0.0550	0.0225	0.0135
Std	**1.0495**	**XXX**	**0.3930**	**0.2775**	**0.1640**
9	0.2885	0.1605	0.0715	0.0330	0.0170
10	0.2920	0.1575	0.0805	0.0260	0.0175

Table 11. Vaccines from poorly maintained pharmacy shops – Mean concentrations

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	**982.225**	**510.28**	**239.735**	**134.84**	**59.5195**
1	174.54	107.855	59.348	23.574	
2	160.575	93.6285	30.1565		
3	150.16	74.1395	21.4095		
4	184.525	111.62	61.3675		
5	161.23	91.85	26.216		
6	173.06	99.449	44.436		
7	155.315	95.13	48.9195	14.848	
8	158.29	79.39799	24.799		

Table 12. Vaccines from well maintained pharmacy shops – Mean concentrations

	1/10	**1/20**	**1/40**	**1/80**	**1/160**
Std	**967.845**	**498.48**	**252.575**	**118.98**	**73.078**
1	177.96	102.87	50.8525		
2	160.335	94.69	25.512		
3	159.025	86.2725	25.512		
4	151.22	97.082			
5	171.495	102.065	44.502		
6	184.815	97.7975			
7	171.845	102.465	39.7345		
Std	**998.945**	**XXX**	**239.15**	**143.2275**	**57.704**
8	62.726	50.6665			

Table 13. Non-heated vaccines - Mean concentrations

	1/10	1/20	1/40	1/80	1/160
Std	**903.75**	**500.025**	**250.005**	**124.835**	**62.844**
1	170.17	76.493	44.7860		
2	153.10	65.7465	41.9375		
3	106.91	66.47	45.608		
4	100.416	47.4195	34.828		
5	170.4	90.2695	45.465		
6	133.135	53.8635	41.953		
7	171.09	112.078	45.4715		
Std	**998.945**	**XXX**	**239.15**	**143.2275**	**57.704**
8	123.855	47.8395			
9	146.365	55.117			
10	129.315	41.1745			

Table 14. Heated Vaccines – Mean concentrations

	1/10	1/20	1/40	1/80	1/160
Std	**986.165**	**476.04**	**270.955**	**123.275**	**55.216**
1	236.21	101.69	55.916	30.5755	24.523
2	185.57	76.8775	41.52	28.075	20.5975
3	133.765	71.346	35.7135	26.0115	20.132
4	145.815	64.268	38.004	22.6855	21.075
5	180.32	90.409	51.0355	30.927	23.8325
6	123.11	61.235	39.159	27.039	21.5525
7	175.72	81.479	44.7515	27.2125	22.0335
8	156.76	69.785	36.842	21.792	20.7545
Std	**998.945**	**XXX**	**239.15**	**143.2275**	**57.704**
9	149.265	55.4705			
10	151.03	53.515			

Table 15. Glycoprotein content of PCECV

PCECV studied (n)	**Mean glycoprotein content in ng / ml (SEM) according to dilution used**		
	1/10 ***p* value**	**1/20** ***p* value**	**1/40** ***p* value**
Obtained from poorly maintained pharmacies (8)	164.7 (4.1) [155.1 , 174.3]	94.1 (4.5) [83.4 , 104.8]	39.6 (5.7) [26.2 , 53.0]
Obtained from well maintained pharmacies (8)	154.9 (13.8) [122.5 , 187.4] *1.0* [a]	91.7 (6.2) [77.1 , 106.3] *0.83* [a]	37.2 (4.0) [23.1 , 51.4] *0.88* [a]

a: compared to vials from poorly –maintained pharmacies

Abbreviations: **n**: number of vials; **SEM**: Standard error of mean

Numbers in square brackets give the 95% confidence interval.

Table 16. Glycoprotein content of PCECV

PCECV studied (n)	**Mean glycoprotein content in ng / ml (SEM) according to dilution used**		
	1/10 ***p* value**	**1/20** ***p* value**	**1/40** ***p* value**
Heated (10)	163.8 (10.3) [140.6 , 186.9]	72.6 (4.9) [61.6 , 83.6]	42.9 (2.3) [39.3 , 46.5]
Non – heated (10)	140.5 (8.2) [121.9 , 159.1] *0.11* [a]	65.7 (7.0) [49.9 , 81.4] *0.23* [a]	42.9 (1.2) [36.8 , 48.9] *0.48* [a]

a: Compared to heated vials

Abbreviations: **n**: number of vials; **SEM**: Standard error of mean

Numbers in square brackets give the 95% confidence interval.

Discussion

In India a large number of people exposed to bites from potentially rabid animals do not receive cell culture rabies vaccines due to financial constraints and therefore still receive reactogenic nerve tissue vaccine. To resolve this problem, the World Health Organization (WHO) recommended the discontinuation of the production and usage of NTV in 1992 and national authorities have demonstrated a commitment to phase out its use in favor of cell culture vaccines [67]. Epidemiological data collected from field studies conducted by the National Institute of Communicable Diseases in Delhi indicate that approximately 2.12 million animal bites occur annually. Of these, 1.1 million patients elect to receive post exposure treatment, 0.45 million of which receive NTV which is given free of charge in the public sector and 0.65 million patients receive cell culture vaccines on a self-financing basis [2]. The main limiting factors for replacing NTV with cell culture vaccines are the limited availability and high cost of cell culture vaccines. Our results show that cell culture vaccines like PCECV and PVRV are available in urban as well as semiurban areas and are being used in rabies prophylaxis regularly. Since isolated reports of rabies vaccine failure are questioning the effectiveness of cell culture rabies vaccines, our study, which evaluates the effect of inadequate cold chain on the potency of PCECV samples, becomes very important not only for the health professionals but also for policy makers with respect to their efforts in rabies prevention.

We conducted a community based study of vaccine storage and handling in pharmacies situated in South India. We selected three adjoining districts from three states in South India. In each district, a taluk head quarters and a district head quarters were chosen for the study, to represent both semi urban and urban systems respectively. Our intention was to generalize the results to these three South Indian states and offer a baseline against which to compare improvements in quality assurance practices. Key risk factors for proper vaccine storage found in pharmacies were the use of old and defective refrigerators to store vaccines, lack of trained personnel, frequent power outage, lack of alternate power facilities, failure to place thermometer in the refrigerator, and failure to monitor temperatures in all vaccine storage compartments.

The findings from our study are consistent with those from other studies that found problems with vaccine storage and handling [16,19,20, 23,24]. In particular, most of the staff responsible for vaccine storage had poor knowledge about maintaining the cold chain and were unaware of recommendations on vaccine storage. Storage of food or laboratory specimens in vaccine refrigerators meant that refrigerators were opened frequently and that both the code of practice for storing vaccines and the food hygiene regulations were broken. Moreover, the findings emphasize the need for daily temperature monitoring in all refrigerators. This is especially critical when refrigerators are older and can no longer maintain temperatures of 2 to 8 ^{o} C. The other interesting observation in our study

was that 16.4 % of the pharmacies accepted that they switch off their refrigerators during the night time. In reality this number could be still high, which might further jeopardize the cold chain system.

Even though we included only the pharmacies storing rabies vaccines for our study, these pharmacies were storing other vaccines as well. Our study indicates that in addition to rabies prophylaxis, other immunization programmes are under the risk of failure due to inadequate cold chain. This lack of attention to correct vaccine storage contrasts sharply with common practices in developing countries, where meticulous attention is paid to vaccine storage [93]. Hence to achieve success in any vaccination programmes, particularly in rural and less populated urban areas, need of the hour is to bring the vaccine storage and transportation policies in line with the current guidelines [13]. This can be achieved through educating the vaccine handlers about the importance and maintenance of cold chain, and strict observation of guidelines from the licensing authorities.

Our study results show that cell culture rabies vaccines are indeed exposed to non-ideal temperatures. Rehan et al [7] while reporting two cases of rabies vaccine failure, observe that the efficacy of rabies vaccination may be high when administered under ideal conditions but its failure rate is considerable in their set up due to many reasons like

repeated power failures and lack of refrigeration facilities in the rural areas leading to reduced efficacy of vaccine, and the tendency of the patients or their attendants to take the vaccines to their homes and store and administer them in an unsupervised manner. Similar scenario exists in our community and it could be the explanation for rabies vaccine failure. However reduced vaccine potency may not be the only cause for vaccination failure as passive immunization with antirabies immunoglobulin is neither advised nor offered by most of the primary healthcare staff. Furthermore, Hemachudha and colleagues [94], while reporting rabies vaccine failure cases from Thailand, mentioned that, rabies vaccine failures may be due to causes other than reduced vaccine potency. However to exclude the possibility of reduced vaccine potency due to inadequate cold chain, a pilot study to assay the potency of rabies vaccines and immune globulins being offered for prophylactic or therapeutic usage in human and veterinary establishments in the affected community is indicated [3]. That would vindicate the authenticity of the two integral components of the therapy against rabies prescribed at various rabies immunization centres.

This proposal has been followed up, in part, in our study by indirectly measuring the potency of PCECV samples collected from the community based pharmacies. Our results showed that potency of the vaccines obtained from the poorly maintained pharmacies was similar to

the vaccines from well maintained pharmacies. Our next set of experiments which aimed at assessing the effect of high storage temperatures on PCECV potency, by simulating those conditions in the laboratory, showed that potency of the PCECV remains intact even after storing these vaccines at non-ideal temperature [37^0C] for 180 hours. These results support the earlier reports depicting the thermostability of PCECV [94]. Our study shows that reduced vaccine potency, due to high storage temperatures and / or inadequate cold chain maintenance during storage and transportation, may not be the cause for rabies vaccine failures seen in developing countries.

Failures of treatment are failure to deliver the three components of post exposure management i.e. wound wash with soap and water, correct and prompt active and passive immunization, or failure of the patient to mount an immune response. For example: if there is delay in starting treatment; failure to complete the course; injections of vaccine or RIG into the gluteals; or immunosuppresion by drugs, HIV, cirrhosis, concurrent treatment with chloroquine or other illness. The other clinical picture that presents as post-exposure prophylaxis failure is development of disease due to a short incubation period. If the animal bites in an area with sparse nerve endings (e.g., the leg), there is a window period of up to a few days between the bite and virus entry into nerve endings. In such cases active immunization alone may suffice. In the recommended vaccination

schedule, antibody production would commence after the second dose, and will be detectable by laboratory testing before day 10. However, if the biting animal was rabid, passive immunization must be given irrespective of the site of bite. The incubation period is too short in case of bites on areas with high density of nerve endings e.g., face, hand, genitalia. Here, viruses may come directly into contact with nerve endings. In these cases in addition to the wound wash and active immunization, passive immunization is mandatory. While soap disrupts rabies virus particles instantaneously by dissolving the lipid coat, rendering them non-infectious, water disperses soap into the wound and also mechanically removes unadsorbed virus particles. If first aid was delayed or unsuccessful, then virus enters and multiplies in the cells, and huge numbers are released into the surrounding tissue. Eventually viruses meet with nerve endings and enter nerve cells. Since each cell cycle of multiplication takes several hours, passive immunization with equine RIG / HRIG prevents the virus entry into fibroblasts, either in the first cycle itself or in subsequent cycles. Thus, virus entry into nerve endings is prevented, especially by the locally injected immunoglobulin [95]. Even though in some cases of rabies vaccine failures passive immunization is given, the details regarding time delay in initiating the wound treatment and initiation of passive immunization is not given. Our study also shows that equine RIG or HRIG is not available in any of the inspected pharmacies indicating that even though active immunization is given promptly passive immunization is not given. The

reasons for this could be high cost and lack of availability of equine RIG or HRIG [96]. Thus the reported cases of rabies vaccine failure are not due to 'therapeutic failure' of 'appropriate' post-exposure prophylaxis, but due to inadequate post exposure prophylaxis. Hence it seems possible that this surreal cell culture rabies vaccine failure is due to delay in initiating wound treatment and inadequate passive immunization and not due to reduced potency of these vaccines. We suggest a community based survey to study the post exposure rabies prophylaxis practices to give more evidence in this regard.

The major limitation with our cross sectional survey is small sample size. Although our study is small, it highlights serious problems with vaccine transportation and storage that could jeopardize the success of an immunization programme. Our inability to use NIH test, due to lack of necessary biosafety infrastructure, to evaluate the rabies vaccine potency is the other major concern. Even though NIH test is the standard test for assessing rabies vaccine potency its shortcomings are well known. Hence we used immunocapture ELISA test as an indirect measure for rabies vaccine potency. Immunocapture capture ELISA is reliable and correlates well with the NIH test [86]. Small number of PCECV were tested for practical reasons. Sample size calculations of PCECV vials for the ELISA suggested large, unfeasible numbers. Hence, we chose the above stated numbers of PCECV vials and planned to do retrospective power analysis to determine whether our study suggests equivalence between the groups.

Statistical analysis later suggested that it is not possible to show significant differences even with large sample sizes.

Conclusions

Our findings show that the cold chain maintenance during transport and storage of PCECV, a commonly used cell culture rabies vaccine in South India, is inadequate and these vaccines are indeed exposed high temperatures. However there was no significant difference in the glycoprotein content, an indirect measure of vaccine potency, of the PCECV samples collected from poorly maintained pharmacies and well maintained pharmacies. Similar results were seen in the heat simulation study wherein the glycoprotein content of PCECV samples exposed to high temperature was similar to PCECV samples maintained at recommended temperatures. Our study indicates that PCECV vaccines are thermostable and that they retain their potency despite inadequate cold chain. We conclude that reduced vaccine potency due to inadequate cold chain may not be the cause for the failure of post exposure rabies prophylaxis. Delay in initiating the wound treatment, inadequate passive immunization, immunodepression, chronic disease, surgery under anaesthesia, concurrent use of alcohol or drugs, and antagonism of vaccine effects by immunoglobulins could be the reasons behind this surreal cell culture rabies vaccine failure.

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Annexure 1.

VACCINE STORAGE PRACTICES IN SOUTH INDIA sl no:

1. **NUMBER OF PERSONNEL RESPONSIBLE FOR HANDLING VACCINES AND THEIR EDUCATIONAL STATUS**.

A. B.

C. D.

2. **REFRIGERATOR**.

A. used / not used. B. make - <5 yrs / >5 yrs

C. condition-satisfactory / unsatisfactory D. store other items – yes / no

E. used round the clock / switched off during some time of the day. ( )

F. max-min thermometer use - yes / no

3. **POWER SOURCE ( )**

A. alternate power source - available /not available.

B. power cut - / day, / week.

4. **TEMPARATURE MEASUREMENT IN REFRIGERATORS**.

A. monitoring-twice daily / once daily / weekly / monthly

B. usual temperature range -

C. temperature recorded during the interview-

5. **VACCINES THEY MARKET**.

	Storage Conditions		**Storage Conditions**
A.		D.	
B.		E.	
C.		F.	

6. **RABIES VACCINE**.

A. brands available-

B. manufacturing date-

C. sales- / week, / month

D. source and transport-