Nine-banded Armadillo Dasypus novemcinctus Animal Model for Leprosy (Hansen's Disease)

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Summary
Leprosy (*Hansen's disease*) is a chronic infectious disease of mankind caused by *Mycobacterium leprae*. Despite several serious scientific attempts, *M.leprae* has never been cultivated in artificial bacteriological media. When *M.leprae* was identified as the causative germ of leprosy, Hansen (who discovered the causative germ of leprosy in 1873) attempted to cultivate the germ in animal models, but he failed. Nearly 85 years after the discovery of *M. leprae* successful transmission and multiplication of *M.leprae* in the footpads of laboratory mice was achieved. The mouse footpad model, although widely used in experimental leprosy, has its own limitations (*Sheppard, 1960; Rojas and Lovik, 2001*). In 1971, significant advancement in experimental leprosy research was made when nine-banded armadillos (*Dasypus novemcinctus*) were successfully infected with *M.leprae* and developed generalised lepromatous leprosy over a period of 18 to 24 months post inoculation. Since then, the nine-banded armadillo has become a favoured animal model for experimental leprosy (*Krichheimer et al., 1972; Job et al., 2003*). They remain as the only immunologically intact species which regularly develops lepromatous leprosy.

Introduction
Leprosy (*Hansen's disease*) is a chronic infectious disease of mankind caused by *Mycobacterium leprae*. Despite several serious scientific attempts, *M.leprae* has never been cultivated in artificial bacteriological media. When *M.leprae* was identified as the causative germ of leprosy, Hansen (who discovered the causative germ of leprosy in 1873) attempted to cultivate the germ in animal models, but he failed. Nearly 85 years after the discovery of the *M. leprae* successful transmission and multiplication of *M.leprae* in the footpads of laboratory mice was achieved. The mouse footpad model although widely used in experimental leprosy has its own limitations (*Sheppard, 1960; Rojas & Lovik, 2000*). In 1971, significant advancement in experimental leprosy research was made when nine-banded armadillos (*Dasypus novemcinctus*) were successfully infected with *M.leprae* and developed generalized lepromatous leprosy over a period of 18 to 24 months post-inoculation. Since then, the nine-banded armadillo has become a favoured animal model for experimental leprosy (*Krichheimer et al.,1972; Job et al., 2003*). They remain as the only immunologically intact species, which regularly develops lepromatous leprosy.

Armadillos are primitive, insectivorous to omnivorous, nocturnal mammals that construct and live in burrows; they are brown or grey in colour and have yellowish white-coloured hairs on the ventral side of the body arising from soft skin (*Fitch et al., 1952*). They have a hard, flexible carapace (shell) formed from ossified (fused) dermal plates on the dorsal side protecting most of their body from head to tail. This bony armour has nine movable rings between the shoulder and the hip shield which gives an armoured appearance hence the name "Armadillo" means "*Little armoured one*" in Spanish. The name Dasypus is derived from a Greek Word

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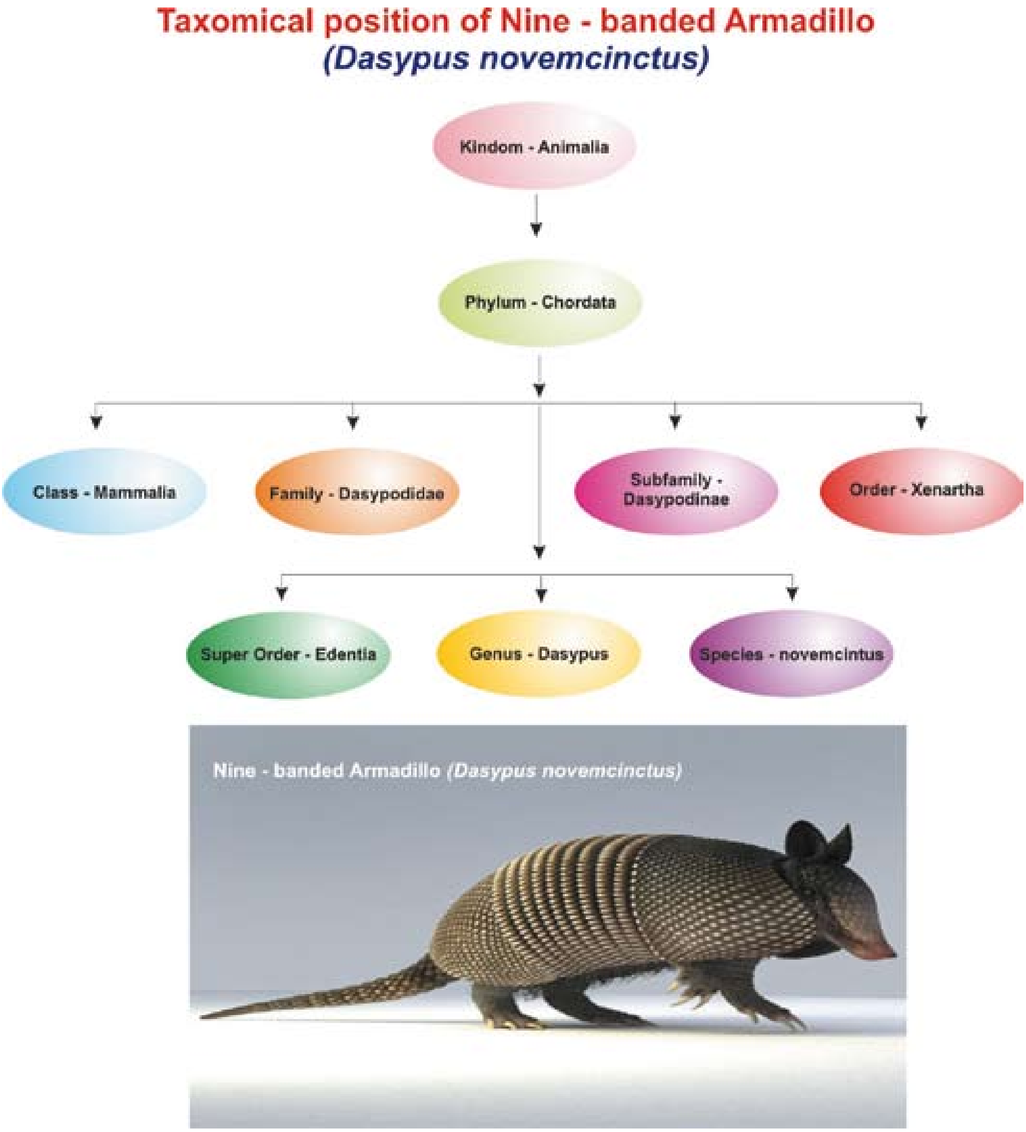
for "*Rabbit*". Combining the word "*novem*" which means "*nine*" and "*cinctus*" which means "*Band*" gives a meaning of "*nine banded rabbit*" and it is stated that a nine-banded armadillo without its shell or carapace resembles a rabbit. Even though it is called nine-banded armadillo, some tend to have between seven to eleven bands depending on their distribution range and location.

The head is small with a long, narrow, pig-like snout. They possess well-developed earlobes, small eyes, short powerful legs, and sharp long claws used for digging burrows in which they live. Armadillos are not blind, but they do have poor eyesight. They have a keen sense of smell in order to find buried insects (their food). They rely on their ears and nose more than their eyes to detect food or predators. The skull is flattened with a long lower jaw (*Mayers et al., 1975*). Female armadillos weigh 3.6 to 6 kg; males are slightly larger and range from 5.5 to 7.7 kg. The total length of the body is 616 to 800 mm; tail length is about 245 to 370 mm, hind foot length 75 to 107 mm, ear length 40 mm, skull length 85.5 to 100 mm.

Although they belong to an ancient taxonomical order "*Edentia*" which means "*toothless*", the nine-banded armadillo has peg-like rudimentary teeth ranging from seven to nine on each side of the upper and lower jaw. They do not have incissors or canine teeth and the teeth are single rooted, and adult teeth do not have enamel. They are solitary animals rarely congregating in groups, sensitive to smell, vibration and noise and they are capable of climbing, jumping and swimming. They squeak or squeal when they feel threatened. They emit a grunting noise while they are foraging. When they feel threatened, they hurry to a nearby burrow. If there is no burrow nearby, they curl up as much as possible to protect their soft undersides. When chased into a burrow they wedge themselves in by arching the back against the burrow walls and are impossible to dislodge. Nine-banded armadillos have four toes on each of their front feet and five toes on their hind feet. The long tapering tail is encased in 12 bony rings that shield the rest of their body. *D.novemcintus* eat a wide variety of food ranging from insects to plants. Although they are primarily insectivorous they eat other organisms like small reptiles, amphibians and even dead birds (*Redford, 1985*). They use their long tongue to get ants, beetles and termites. In a study conducted during 1954 on the stomach contents of nine-banded armadillos, researchers were able to identify that a major portion was of animal origin, followed by food originated from plant, arthropods and small vertebrates. When they are housed in captivity they are fed with cat chow containing protein and mineral mixer (*Truman, 2005*).

Armadillos can occupy a diverse range of ecological habitats, but are usually found in close association with water supplies (they live close to water bodies). Armadillos are known for digging sizeable burrows close to the trunks of trees, as well as in the banks of streams or in limestone cliffs near surface water supplies (*Wolfe, 1968*). They construct numerous, complex burrow systems by digging with their claws; sometimes they share the burrows with other animals, like skunks and rabbits. Armadillos tend to have more than one burrow some being active and others are forgotten. These burrows are anywhere from four to twelve feet long and tend to join at a central den. Armadillos are good swimmers. They can even go quite a distance underwater and are capable of walking along the bottom of streams and ponds. When confronted by a water barrier, armadillos have two means of crossing. If the water is shallow and narrow, armadillos can walk or run across the bottom because of their capacity to build up a large oxygen debt (they can hold their breath for four to six minutes at a time). On approaching a large body of water, it can lower its density by gulping in air, which inflates its stomach and intestine, and enables the animal to float and swim across like a dog (*Frapel et al., 1998; Boggs et al., 1998*). The ability to cross streams and rivers might have helped armadillos expand their home range. Because of their almost complete lack of hairy covering, armadillos are easily affected by climatic conditions. In summer season, they are more active



in the evening and at night but in midwinter their daily activities are reversed and the animals become active during the warmest part of the day, usually in mid-afternoon (*Smith & Robin, 1984*).

Zoo-geography
D. novemcinctus is not territorial. In the USA armadillos are unevenly distributed all over the southeast. Armadillos are not native of the USA, but began slowly expanding their range north from Mexico around the 1880's (*Taulman & Lynn, 1996*). Of the twenty species of armadillo, only the nine-banded armadillo appears to be increasing in number. Presently nine-banded armadillos inhabit eight states in the U.S.A: Texas, Louisiana, Arkansas, Oklahoma, Alabama, Mississippi, Florida,

and Georgia (*Smith, 1984*). They are also present in Caribbean islands like Trinidad and Tobago, and were probably carried there aboard trading ships (*Humphrey,1974*). Scientists have discovered that nine-banded armadillos evolved or replaced the extinct species of giant armadillo D.bellus that once roamed in South America more than 10,000 years ago (*Nixon,1995;Stuart,1986*). Estimated statistics reveal that the current population of D. novemcinctus is between 30-50 million (*Gilbert et al., 1995*). Armadillos have a very low metabolic rate, which means they do not produce much body heat and thus are not good at living in cold areas. The main factors limiting their ecological distribution range appear to be associated with their poor tolerance to cold temperatures, and the capacity of the environment to produce sufficient insects, the mainstay of their omnivorous diet (*Talmage& Buchannan, 1954*). Since they do not have a lot of body fat, they forage for food on a daily basis. Just a few cold days in a row can be deadly to armadillos. Wild armadillos are caught using a long-handled nylon net and brought to the animal care facilities for experimental purposes. They are housed in brick pens measuring 8 x 8 feet. They are provided with clean drinking water ad libitum. Bottles with sipper tubes can be used for this purpose after an adaptation period. Alternatively, drinking water can be provided in bowls kept in the corner of the pens. As many as 4 to 6 animals can be housed in one brick pen (*Truman et al., 1991*).

Husbandry and Handling

Armadillos are very sensitive to temperature. Room temperature should be maintained between 24 to 27°C. During winter plenty of nesting materials should be provided or otherwise room heaters must be used to keep the room warm (*Wamper, 1969*). The brick pens must be cleaned at least once a week. The bedding materials are cleaned and changed at this time. Pens must be cleaned with detergent germicide (wide spectrum antibiotics of phenolic derivatives). It is easy to handle armadillos with some precautions. They generally do not bite while handling them but they can inflict serious scratches to the handlers with their powerful limbs and sharp claws. Strong rubber gloves should be worn for handling. It is preferable to pick the animal up by the sides of the carapace. When this is not possible, the animal can be picked up by its tail. The tail should be grasped as close to the body as possible to prevent injury to the tail. The animal's weight should be supported by placing one hand at the back while picking up the animal by the tail. Handling of advanced pregnant females should be avoided as much as possible. Male and female armadillos cannot be easily distinguished by visual examination. The adult male is a little larger in size and weight. Females possess a uro-genital sinus serving both as vagina and urethra. This sinus opens to the outside through a long cleft. The male reproductive system is typically mammalian, but there is no scrotum. The testes descend into the pelvis. The penis is prominent and can be easily identified.

Reproduction

Armadillos are believed to pair for each breeding season, and a male and a female may share a burrow during the season. Because of the bony carapace and ventral position of the genitalia copulation occurs with the female lying on her back. The armadillo's lifespan can range from four to seven years, but it has been known to survive up to ten years in captivity. Armadillos do not breed well in captivity. This problem appears to be due to abnormal hormonal levels in females and failure to ovulate in captivity. Armadillos are monoestrus, monovular, polyembryonic mammals which always produce monozygous quadruplets; they are the only mammals which can consistently give birth to four babies of the same sex. A litter typically consists of four genetically identical baby armadillos of the same sex which develop from a single fertilized egg (*Enders, 1966*). They exhibit both gestational diapause and polyembrony. Implantation of the fertilized egg may be delayed for up to fourteen weeks after conception (*Smith & Robin, 1984*). In the wild, mating takes place between July and August, but implantation is delayed until November. Parturition takes place about 4 to 5 months after implantation which means after fertilization, the armadillo embryos undergoes a 4 to 5 month period of arrested development prior to implantation, after which it immediately divides to form four identical offspring of the same sex (*Szabuniewicz & McCrady, 1969; Hamilton & Whitaker, 1979*). The delay to implantation of the embryo in the uterine wall is thought to be caused by stress (*Watson, 1989*). The gestation period is around 120 days and the young are weaned at the age of ten weeks (*Job et al., 1984*). Armadillos are born with their eyes open and begin to move around within a few hours. The nursing period is probably less than 2 months, but the young may remain with the mother even after weaning until they are several months old. At about 2 to 4 months of age the baby armadillos are ready for weaning and leave the burrows to forage for food (*McDonough et al., 1998*). Within 3 to 4 months after birth, the young are independent (*Pepler et al., 1986*). Sexual maturity is attained after about one year. Normally the young born in one year mature during the winter and mate for the first time in the early summer of the following year. Even though the nine-banded armadillo produces one ovum per year, polyembryony contributes to their high rate of reproduction (*Watson, 1989*).

Leprosy in armadillos

Leprosy was unknown in the New World prior to immigration of European settlers. Walsh and colleagues proposed that armadillos might have acquired leprosy infection from untreated human patients in the USA sometime after the animals expanded their range in the USA (*Walsh et al., 1977; Stalknecht et al., 1987; Howerth et al., 1990*). Armadillos are highly susceptible to leprosy infection (*Storrs, 1971*). A naturally occurring leprosy-like disease in armadillos has also been reported (*Binford et al., 1977; Walsh et al., 1981*). The exact mode of transmission of leprosy in humans and armadillos is not known (*Storrs & Burchfield, 1985*). When armadillos were found susceptible to leprosy infection many species of armadillos were investigated. In addition to nine-banded armadillos, other species such as the seven-banded and hairy armadillos have also been found susceptible to *M.leprae* infections (*Wheeler & Bharadwaj, 1983*). When brought from the wild for experimental purpose to the animal care facilities, they should first be kept in quarantine and examined for the presence of AFB: Acid Fast Bacilli (*M. leprae*) from nasal smears and skin smears from the ear lobe. It is thought that leprosy attacks the armadillo because of its low body temperature (*Storrs, 1982*). The animals found in Colombia, Venezula and Paraguay are reportedly free from infection (*Innami, 1978; Acha et al., 1988*), but leprosy is known to occur among armadillos in Argentina (*Martinez et al., 1984*). Nearly 85 % of nine-banded armadillos have been reported to get extensive type of leprosy (borderline type of leprosy to lepromatous type of leprosy), whereas the remaining15% are totally resistant. *M. leprae* resistant armadillos can serve as a model for understanding the mechanism of resistance and possibly the genetic linkage. A study with three experiments, using different routes and doses of infection, conducted on 42 armadillos, (*Job et al., 1985*) showed that thirty-six animals developed generalised disease. There was no significant sex or age difference in susceptibility. It was noted that it is quite possible that in armadillos the resistance to the disease is partly genetic. Route and dose of infection make very little difference in the disease prevalence except that intravenous administration of a large dose reduces the period of development of generalised disease. Although a majority of armadillos developed lepromatous disease, borderline leproma is fairly common. The infection in armadillos shows a spectrum of the disease almost like human disease. Smith and co-workers also noticed a tendency for prevalence rates to increase from north to south of the United States along the Texas coast, and suggested that armadillos may have carried leprosy with them from Mexico (*Smith et al., 1983*). The time between infection and development of symptoms in nine-banded armadillos is six months to four years, as opposed to three to six years in humans (*Storrs, 1982*). Armadillos develop severe disseminated leprosy when inoculated with human leprosy bacilli and they serve as a sole source of *in vivo* propagation of *M.leprae* outside the human body (*Kirchheimer et al., 1971*). As few as 10^3 *M.leprae* in an inoculation can infect armadillos. A suspension of *M. leprae* (inoculum) is achieved by doing a biopsy from a heavily infiltrated area of leprosy lesions: like a hypopigmented patch or nodule in the skin of a leprosy patient. After removing the epidermis and fat, the dermis is minced thoroughly and a homogenate suspension is prepared. The enumeration of bacilli in the homogenate suspension is then made using a suitable counting technique and it is ensured that the inoculum contains the minimum infective dose. The incubation period of *M.leprae* in armadillos is as short as ten months and as long as four years. If the inoculum size is 10^8, animals develop heavy infection and a bacterial load of 10^{11} M.leprae per gram of tissue can be harvested from infected animals; this phenomenon has made armadillos an excellent source of propagation of M.leprae outside the human body. The time required to achieve peak infection varies from 6 months with 10^9 inoculation of *M.leprae* and 4 to 5 years with 10^3 organisms. Inoculation is done through the saphaneous vein, and reticulo endothelial tissues immediately take up the bacilli (*M.leprae*) which can slowly proliferate and disseminate to other parts of the body like ear, nose, tongue and lungs. Blood samples can be collected from subclavian, saphaneous or jugular vein in sedated animals (*Moore, 1983*). 2 to 4 ml of blood can be sampled from the saphaneous vein, which is located in the inner rear leg, with the help of a 0.7 mm x 15 mm needle in a 10 ml syringe; the sample so obtained is processed separately according to the type of investigation required (*Ramsey et al., 1981*). Two anaesthetics have been found to be satisfactory. Ketamine hydrochloride (500 mg/kg body weight) intra-muscular has been used and in general it is satisfactory. Ketamine hydrochloride combined with acepromazine maleate (dosage of 4.5 mg / kg ketamine plus 5.0 mg acepromazine maleate) is found satisfactory. Other anaesthetics that are used are fetanyl citrate along with droperidol (*Fournier et al., 2000*). While administering any anesthetic, the animal should not be fed prior to experimental procedures. Care should be taken to place the animal on its side for recovery in a warm location.

Experimental Biology

In the experimental disease, dissemination of infection has been observed in the skin, bone marrow, liver, spleen, lymph nodes, lungs, oesophagus, which are usually observed in human leprosy (*Job et al., 1985*). The degree of lepromatous leprosy in infected animals is non-erythmatous, lepromatous nodular form of the disease containing a large number of *M.leprae* in fourteen months. In view of the importance of nine-banded armadillo skin in leprosy research (*Zhou et al., 1996*), ultrastructural studies have identified three basic cell types of human epidermis in armadillos, ie keratinocytes, melanocytes and langerhan cells. In armadillos the role of langerhan cells and changes in the number and structure are highly relevant to the study of leprosy. Examination of livers from armadillos inoculated with 10^7 *M.leprae* revealed apparent infiltration by bacillated macrophages together with Kupffer's cells containing *M.leprae*. These lesions can be compared to the severe form of (lepromatous) leprosy in humans. The liver cells heavily loaded with *M.leprae* develop a pale granular cytoplasm which became foamy in the late lesions (*Job et al., 1978*). *M.leprae* is also demonstrated in liver parenchymal cells (*Job et al., 1985*). In a study conducted in the United Kingdom, bacilli were found in large numbers in virtually all tissues except the lens, retina, optic nerve, and aqueous and vitreous humour, but the uveal tract was heavily involved. These findings, together with the role of immune-complex in leprosy patients with severe infections, may be helpful in understanding the factors that favour localisation and multiplication of *M.leprae* in the eye (*Hobbs et al., 1978*). In a USA-based study, *M.leprae* lesions were found in cornea and retina and infiltration of

M.leprae inside the macrophages was noticed. The infection of intraocular tissues in armadillo eyes seemed to be mainly haematogenous. Inflammatory reactions were found in eyelid, including the orbicularis muscle, extraocular muscles, tear gland and *Harder's* gland. In animals with severe leprosy the whole uvea was densely infiltrated with foamy macrophages intermingled with small amounts of lymphocytes, plasma cells and neutrophils (*Brandt et al., 1990*). The involvement of peripheral nerves in armadillos can certainly be correlated to that of humans. Clear involvement of ulcerations and loss of sensitivity of extremities are evident. Peripheral nerve neuropathy in armadillos serves as a tool for nerve conduction velocity studies and the results are comparable with those of humans. Immunological investigations of experimental infections could help us to understand the basic susceptibility to leprosy and will help to initiate research in new avenues of immuno- and sero-diagnostics including T cell-based assay designs for early diagnosis of leprosy and leprosy-induced complications (*Purtilo et al., 1975*). The nine-banded armadillo has become the principal source of M. leprae because the body temperature of the armadillo (which ranges between 32 to 35º C) is low enough to favour growth of the bacterium. They serve as a model in biochemical and immunological and vaccine research. The long lifespan of armadillos is helpful for studying the chronic infection of leprosy. Armadillos pick up leprosy infection easily but the disease (natural infection with *M.leprae*) among the nine-banded armadillo is usually severe and sometimes fatal. The time between infection and development of symptoms in nine-banded armadillos is between six months to four years, as opposed to three to six years in humans (*Storrs, 1982*). Although *M.leprae* is cultivable in armadillos the source of infection, mode of transmission, patterns of exposure and the early pathogenesis of infection remain poorly elucidated. The very slow rate of multiplication of *M.lepare*, the long incubation period, the variable host immune response and low incidence in humans make it difficult to link exposure and disease in humans. However, the transient presence of *M.leprae* in the respiratory tract of leprosy endemic communities is comparable with the infection pattern in armadillos. Although more than three decades have passed since armadillos were shown to be susceptible to *M.leprae*, their importance as model hosts in the study of leprosy is just beginning. Preparations of armadillo-derived *M.leprae* used in vaccine trials were analyzed using a combination of morphological, chemical and immunological criteria. Recently the human genome consortium completed a 6 x coverage sequence of the armadillo genome and has begun to annotate sequence data in comparison to humans (*Adams et al., 2005*). This new genomic data may spell out a vast number of biochemical and immunological reagents and will likely facilitate more extensive use of armadillos in other research studies. The international union for conservation of nature and natural resources listed armadillo as endangered species and steps are taken already to conserve them (*Novak, 1999*). Necessary GLP (Good Laboratory Practice), SOP (Standard Operation Procedures) and animal ethics procedures as recommended by the statutory regulatory authorities/ethics committees are already in practice while performing experiment with armadillos.

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