CHAPTER V

DISCUSSION

PART-I EPIDEMIOLOGY
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I. EPIDEMIOLOGY:

Rabies is an endemic disease in the country and the main reservoir of infection is dog. The population of stray dogs in cities as well as rural areas is considerable. In cities the Municipal Corporations maintain a dog squad to catch stray dogs but they are not destroyed because of social and at times religious pressure groups working in a society. Thus these dogs ultimately find their way back in their own locality.

The Ahmedabad Municipal Corporation maintains a dog squad and they catch dogs from city. To study the carrier state of rabies among stray dogs, Municipal Corporation of Ahmedabad city was approached and they provided the dogs caught from city area.

The city is divided in three distinct areas. The area west of river is mostly a residential area with commercial zones and offices. The population residing in this area is comparatively educated and economically well placed. There are pockets of slums in this area where stray dogs are found. Population of pet dogs is mostly found in this part of the city.

The area between east of river and west of railway station is old city with narrow lanes, markets, old residential houses and several offices. There is a considerable stray dog population in this area and pet dog owners are also found.
The eastern most part of the city is industrial area and slums are many. Stray dog population is more.

Totally 226 dogs were collected from the city and nearby areas. These dogs were kept for observation for 11 days for any signs of illness and were sacrificed. Blood serum, cerebrospinal fluid (CSF), brain and salivary glands were collected.

The brain and salivary glands were tested for presence of antigen by impression smear staining by Seller's stain and by inoculation of the material in three weeks old and suckling mice. They were also tested by gel-diffusion technique and complement fixation test. In none of the samples presence of antigen was detected, as can be seen from Table-I.

The infection of rabies is through bite of a dog. The dog may appear healthy and with no sign of rabies. Every bite of a dog is regarded as potentially dangerous and antirabic treatment is necessary. There are reports of healthy dogs excreting virus in saliva (Andral and Serie, 1957; Veeraraghavan, 1970; Fakato, 1975). Yorkovsky (1962) have reported transmission of rabies by normal dogs. Held et al. (1967) were able to establish source of infection in only 63 per cent of cases.

The incubation period of rabies is highly variable from several days to few years. Therefore at the time of bite if we catch a dog and keep it under observation, it
may not be possible to isolate virus by the methods now available. The presence of infection at the time of bite can only be demonstrated by the emergence of overt disease. This is considered as a latent infection by McDermott (1959). "Abortion" refers to cessation of adverse effects of virus upon the host. However, latency and abortion are not clearly distinguishable from masked, inapparent, chronic and recrudescent infection, long incubation periods, eclipse and the carrier state (Walker et al., 1958).

Such inapparent, abortive, masked or latent infections can be reactivated by stress like overcrowding (Soave, 1964), or by inoculation of ACTH (Soave et al., 1961) experimentally in laboratory animals. Such things can happen in nature under stress conditions as stated by Soave (1964) and rabies virus may be reactivated.

Such a thing happening in nature cannot be ruled out. It is true that all bites do not result in disease. Probably long incubation period in rabies can be explained on the hypothesis that a host may be harbouring inapparent infection which might have been reactivated due to stress in his normal life or by taking such medicines which may help in reactivation of infection.

A dog whose bite to another dog had resulted in rabies was caught and kept under observation. The dog was under observation for 302 days. During the period of observation, the saliva was monitored regularly for presence of rabies virus and serum was also collected for detection of rabies
neutralizing antibodies. Rabies virus was not found in saliva by mouse inoculation and antibodies in the serum were not detected by CFT and neutralization test. The dog died of acute gastroenteritis. Similar studies on dogs have been carried out and are carried out at several places. 

Veeraraghavan (1970) reported rabies virus excretion in saliva on 14 occasions in a period of 913 days observation. After death rabies virus was not found in brain, salivary glands nor in any other tissues.

However, a healthy carrier state seems to have been documented in Ethiopia, where five stray unvaccinated dogs were found to excrete rabies virus intermittently over long periods (three to five years) of time (Fakadu, 1975).

In our case the dog never excreted the virus in saliva, nor antibodies were detected in the serum. Brain and salivary glands were found negative for Negribodies on postmortem examination. The past history of the dog, whether the animal was vaccinated previously or not was unknown. The animal was kept in isolation box. The stress factor of any kind was not used on the dog during the period of observation. From the literature it can be presumed that such carrier state does exist in dogs, that they may not show detectable antibodies in their serum, nor Negribodies in brain and salivary glands after death.

In studying the carrier state among stray dogs as in this case, the period of observation should be long enough
of about three to five years as stated. But the dog died due to acute gastroentritis by 302 days. Therefore, a negative finding does not rule out the possibility of a carrier state.

Serum and cerebrospinal fluid collected from 214 stray dogs were examined for the presence of rabies antibody by complement fixation test, and gel diffusion test. 12 sera samples were positive and all 36 CSF samples were negative.

The antibodies naturally occurring in animals usually living free natural life, either in stray dogs, wolves, bats or any other domestic animals have a very low titer, insufficient to protect against a moderate challenge of virus.

Demonstration of presence of antibodies against rabies in dogs, wild animals and rodents in endemic area is an evidence to suggest that exposure to rabies virus is more common in nature than previously believed (Doege and Northrop, 1974). Various forms of rabies virus infection occur under natural conditions and further research is necessary and desirable on abortive and symptomless infections (Gribencha, 1976).

Polson and Wessels (1953) were first to report that rabies virus infected cells produce complement fixing soluble antigen, and Villemont and Provost (1958) were the first to show presence of two precipitable fractions in tissues containing rabies virus. Complement fixation reaction was found positive in all animals in which Negribodies were present and also in animals where Negribodies were not demonstrable (Linsert, 1959).
Grasset and Atanaslu (1961) found immunoprecipitation reaction giving good results, and can be regarded as a complementary diagnostic aid (Grasset, 1967). However positive results are of value (Kolomankin, 1966), but negative results should always be considered doubtful and other confirmatory tests should be performed in parallel, since these tests were not considered satisfactory for routine diagnosis (WHO, 1966).

With street virus infection, CF antibodies appear first by six to seven days and in an experimental infection in mice with street rabies virus it was seen that CF tests and SN tests were jointly positive (Baczynski, 1963). The rapidity, accuracy and safety of CFT recommended it for routine use (Wolter, 1964).

There is a direct relationship between the measurable complement fixing and protective activities of rabies virus as long as immunizing surface protein remains unaltered. If the surface structure is disrupted beyond a critical level only CF activity can be measured (Schneider, 1971). The neutralizing antibody found in both IgG and IgM appears to protect against a subsequent challenge with rabies virus. The envelope glycoprotein of the virus induces neutralizing antibody and elicits a protective response. When purified nucleocapsid is inoculated into rabbits, complement fixing antibodies are produced. Thus serologic tests have been found valuable tools for epidemiological studies for the purposes of measurement of circulating antibodies in a population. Complement fixation and gel diffusion tests
were used for its rapidity, accuracy and safety and further to avoid use of large number of laboratory animals.

Out of 214 sera samples, 12 sera were found to contain antibodies to a detectable level in stray dogs (5.36 per cent). 36 CSF samples were all negative. Afshar et al. (1972) found precipitating antibodies in sera of stray dogs and camels, and observed that inapparent or abortive infection with rabies virus may occur in dogs. Nanavati (1973) observed neutralizing antibodies in 2.0 per cent sera of stray dogs. Ferro et al. (1974) found this incidence 3.9 per cent. Afshar and Bahmanyar (1978) reported incidence ranging from 2.0 per cent (Nanavati, 1973) to 13.9 per cent (Bell et al., 1972) and 18.3 per cent (Yasmuth et al., 1974), of serum samples from apparently healthy stray dogs from the literature reviewed. Thus the results of this study correlates well.

CSF usually does not contain antibodies against rabies. However a high rise of CSF antibodies are seen in prolonged clinical illness and recovered cases. (Hattwick et al., 1972; Tillotson et al., 1977b).

The presence of antibodies against rabies in stray dogs indicate that either the dogs had been asymptomatic carriers and/or they had an exposure to antigenic stimulus in mild manner, infection being not enough to cause the disease.

Clinical disease results by combination of host factors, pathogen factors and environmental factors. For the perpetuation of disease, the dose of the virus should be
sufficient and the virus should not be destroyed by the host by its natural body resistance. It is possible that virus may survive in body at certain sites like brown fat in case of bats and it may be excreted in saliva, urine etc. This state is called as carrier, asymptomatic, inapparent or latent state.

When an antigen is present in the body it naturally produces antibodies. If the stimulus is in form of a low dose the rise in antibody titer is also low, and subsequent larger dose may result in disease if reinfection occurs. In nature, environmental conditions change and may cause stress which ultimately may reactivate an infection resulting in clinical disease.

Environmental factors change occasionally over a long period of time in nature to which animals living free natural life are exposed. The rabies virus has been found occasionally in saliva of healthy dogs, and various organs of foxes, brown fat in bats and in other animals leads one to believe that rabies virus can remain in a latent or inapparent form or inactive in the body of the host over a long period of time. Therefore under natural conditions reactivation of infection is possible. Stress factor can play an important role in perpetuation of disease particularly in human beings, as they being social animals. The presence of antibodies due to such first infection is not enough to protect the animal against subsequent reinfection or reactivation.

The presently available methods for detecting virus antigen may be inadequate to detect the presence of very
small amount of virus. Electron microscopy of every brain or suspected tissue is not possible. Therefore the only method at present in use is to find out the detectable antibodies against rabies in a host, thereby getting indirect evidence of previous exposure or infection. In an endemic area, this is probably the best method for getting indirect evidence of the disease, which has been proved by several such studies in rabies endemic area.

Similarly serum samples were collected and information regarding vaccination was obtained from pet dogs and pet dog owners respectively who were reporting for prophylactic antirabic vaccine. All the pet owners were well informed about rabies and every body reported within six to nine months for revaccination. 17 pups of five to six months age were presented for primary vaccination, and only seven young dogs over six months of age were brought for primary vaccination.

This study was carried out to find out the danger of contacting rabies to pet dog owners through their pets, and to find out the correlation of similar results obtained in stray dogs in case the pet animals are left unvaccinated in adult age or are irregularly vaccinated. It is observed that out of the 214 sera samples examined, 190 had detectable levels of rabies antibody and 24 had no antibody at all. These 24 sera were from 17 pups and seven young dogs which were presented for prophylactic antirabies vaccination for the first time.
This indicates that there is a considerable amount of antibody, definitely a response of previous vaccination, is present in pet dog population of the city and that bite of these animals may not result in infection and disease, provided vaccination at regular interval is carried out. Raichowdhuri *et al.* (1973) observed a fatal case in pet dog owner and stated that his pet dogs were probably not responsible for causing hydrophobia.

Concurrent serological studies for detection of presence of rabies antibodies were conducted on sera samples collected from human beings reporting for post bite antirabic treatment. A questionnaire was prepared requesting them to provide necessary information which was used for further analysis.

This population was considered as exposed to risk, either due to their profession or way of living. Ruegsegger *Brodsky*) *et al.* (1961, & have shown neutralizing antibodies in sera of veterinarians and cave explorers, who because of their profession are exposed to infection. In a city of about 22 million population, number of people reporting for ART appears small. It can be safely assumed that there is much underreporting, which has been observed by Maheshwari and Sharma (1972). Persons might not report for vaccination as the schedule is long running upto 14 days, there is a fear of post vaccinal complications, and false sense of security that bite from rabid dog only will result in disease and not of a healthy dog.
Of the 572 human sera examined for presence of rabies antibody by complement fixation, only 11 sera were positive (1.92 per cent). Only three sera samples (0.52 per cent) at 1:2 dilution fixed 100 per cent complement. 75 per cent complement fixation was observed at 1:2 dilution in eight sera (1.398 per cent) and at 1:4 dilution in three sera (0.52 per cent). 50 per cent complement fixation was observed at 1:4 dilution in eight sera and in 1:8 dilution in three sera. All sera did not fix complement at 1:16 dilutions. The results are presented in Table-VI.

Such a low level of rabies antibody in human population indicates that the population had a previous contact of rabies antigen. This is quite possible in an endemic area, and the results correlate with such endemity shown by the presence of rabies antibody in stray dog population of the same area.

In case of dogs 5.36 per cent of the samples showed positive results, where as in humans only 1.398 per cent showed such results and that too at last dilution of 1:8, which in case of dog sera is 1:16. Thus it can be assumed that human beings are not so much exposed to rabies infection as compared to dogs. However, reactivation of previous exposure cannot be ruled out if precautions are not taken. The very long incubation periods reported in literature can be attributed to such occurrences in nature, where infection cannot be traced. Raichowdhuri et al. (1973) reported a fatal case of a man who had no contact of rabid animal in last three years except his two pet dogs who were probably not responsible for causing hydrophobia.
Afshar et al. (1972) examined 91 human sera for presence of rabies precipitating antibody and found all of them negative. The number of samples studied are small for epidemiological investigations of a disease in a population of species which is moderately susceptible and accidental host.

However, carrier or abortive state in case of human beings have not been studied so far like it is studied in dogs in an endemic area. At Central Research Institute, Kasauli (H.P.) humans accounted for 1.3 per cent out of the 23 species which were found to be the source of exposure to the patients (Thomas, 1973). What were the reasons to consider 1.3 per cent of humans as a source of exposure is not mentioned.

These 572 persons who reported for ART, were grouped in six age groups by sex to find out the incidence of dog bite age and sex wise. The results are presented in Table-VIII. Totally 448 males (78.32 per cent) and 124 females (21.68 per cent) received treatment. The highest rate of bite was in group II representing age group of three to 12 years (38.11 per cent). The next group III (13-24 years) constitute 26.22 per cent with 150 bite cases. Close to this is group IV (25-45 years) with 147 bite cases (25.7 per cent) group V (46-65 years) accounted for (7.52 per cent) 43 cases. The group I (0-two years) and group VI (above 65 years) accounted for eight cases (1.398 per cent) and six cases (1.048 per cent) respectively. Males outnumbered females in every age group.
It is observed that high risk group are the persons who are between three and 12 years, all of them school going and not adult enough to protect themselves, and at the same time fond of playing and teasing with dogs. The next two vulnerable groups are between 13-24 years and 25-45 years. These groups are composed of either school or college going youngsters and working class of people. These people have an outdoor life, exposing them to episodes of bite. The group of infants (0-two years) and old (above 65 years) are mostly found indoors not exposing to outdoor life and thus the incidence of bite in these age groups is very low 1.39 per cent and 1.048 per cent respectively, which is expected.

The difference in various age groups between males and females does not seem significant except in group I (0-two years) and group VI (above 65 years). This is expected as female babies are not prone to move out or allowed to as much as male babies, and old women have mostly indoor life, they have certain fixed places to visit, and they by nature are very cautious.

The monthwise distribution of bites in males and females in six age groups is reported in Table-IX. It is observed that highest bites 123 are recorded in May (21.5 per cent), followed by March, 111 (19.4 per cent), October 83, (14.5 per cent) and February 66 (11.53 per cent). In June there were 57 bite cases (9.965 per cent) and in July and August no bite cases were reported. Rest of the months had less than five per cent bite rate.
The factors responsible for dog bites in males and females in six age groups reveal that maximum number of bites 130 (22.72 per cent) resulted when persons visited unknown locality. Next in order are teasing the dog (114), going out for work (99), school going (76), cycle accidents (58), while playing (40) handling pups (26) and pet owners and their servants and others having contact with dogs accounted for 29 cases. None of the person reported a bite attributable to a rabid dog.

The bites were classified as class I, II and III. It is noted that (84.26 per cent) 482 were class I bites and 90 (15.74 per cent) were class II bites. None of the bite was class III (Table-XI).

Table-XII represents parts of the body bitten by dogs. Bites on leg are 376 (65.73 per cent), 163 (28.50 per cent) on hands, 22 (3.85 per cent) on abdomen, seven (1.22 per cent) on back and only four (0.67 per cent) on head and face.

The bite rate is higher in males than females and the difference is 3.6 times indicating vulnerability of males to dog bite is 3.6 times higher than the females. This is a wide ratio as compared to observations made by Parish et al. (1959) in Pittsburgh. But in this part of the country females don't live much of outdoor life, except for going to school and work.

The difference in the incidence of bite between males and females in all age groups except group I (0-two years)
and group VI (above 65 years) is not significant. In I and VI age groups this significant difference is explained before.

The school going children and the working people with life outdoor/are more prone to bites. Children play with dogs, tease them and move out for better part of the day. Traders, howkers and routeman are moving frequently in unknown locality and get bite. Similarly working females are exposed. Thus it can be understood that these school children, school and college going boys and girls, who have an outdoor life, play games, move about in various localities are high risk groups. It is also observed that too young and old people have less chances of getting bite, and age is a factor in incidence of bite and so is sex as can be seen from results. This is in disagreement to the observations made by Shah and Jaswal (1976), but agrees with the observations of Parish et al. (1959), Rhodes and Rooyan (1962), Schawabe (1969), and Maheshwari and Sharma (1976).

The month of May recorded more than 20 per cent of total bite cases followed by March 19 per cent, October and February. This indicates seasonal variation. The observations made in this study correlate well with Schawabe (1969).

Maetz (1979) have also observed increase in frequency of animal bites in spring and summer months in USA.

The maximum bites were on legs and hands of the persons bitten, which is about 94.23 per cent. Rest of the part of
the body like abdomen, back, head and face are not frequently exposed to bite unless the animal attacks the person. Such things happen to persons when the animal is teased or during trespassing an area where watchdogs are kept. Leg is the usual site for dog bite as it is at the height of a mouth of dog so easily accessible. Hands get bite during playing with dog or teasing or protecting the self against a biting dog. The situations considered by Addo (1977) compares favourably with these observations in case of teasing and playing and such conditions which can be considered a provoked attack. Cyclists may drive over or near the dog and get a bite.

The bite at extremities are more common and frequent. Cochary and Davies (1960) reported 85 per cent of bite on extremities and Schawabe (1969) 71 per cent. Parish et al. (1959) reported 76 per cent bites on extremities. In this study 94.23 bites are on extremities and comparatively less percentage of bites on other parts of the body. However, the results compare fairly well with reported observations.

The classification of bite reveal that 84.26 per cent bites were classed I and 15.74 per cent bites were classed II. Along with this data a comparison is necessary about the number of inoculations taken by a person receiving ART. It is observed that 88.65 per cent took seven or more inoculations, and 11.0 per cent took less than seven injections. Only two (0.35 per cent) completed course of 14 inoculations.
These observations lead to the simple conclusion that persons who are enlightened enough to present themselves for ART give up half way. Though it is believed that in class I bite seven inoculations are sufficient, in practice a full course of 14 inoculations is recommended. Massive under reporting and high defaulter rate in ART calls for education of the people. The situation in Africa reported by Oboegbulem (1978) is less enlightened populations, ignorance and mass illiteracy and lack of informative education are equally applicable in our country.
II. DIAGNOSTIC METHODS:

The histologic diagnosis of rabies evolved in three steps. The first was the histologic study of the neuraxis in man and animals succumbing to rabies and the description of specific lesions called Babes nodules (Babes, 1892). Research then concentrated on proliferative and infiltrative lesions (Van Gehuchten and Nelis, 1900), but they were soon found non-specific.

The second period began with the description by Negri (1903) of inclusion bodies in the cytoplasm of nerve cells infected with street rabies. These bodies were termed as Negribodies, and the presence of these bodies was used as a practical diagnostic test for rabid animals and laid the ground for the long used test, which holds good even to-day.

In earlier research and diagnosis, the rabbit played an important role (Galtier, 1879, Pasteur, 1881), and later on mice (Hoyt and Jungleblut, 1930). Webster and Lawson (1935) developed a standard mouse inoculation test for the diagnosis of rabies, and it was found that specimens found negative by various staining techniques were found positive by mouse inoculation.

Sellers (1927) established a rapid staining technique for detection of Negribodies. Prior to that modifications of Mann's method were being used. Various modifications of staining Negribodies were evolved. The Negribodies could be stained by various dyes and the most important factor was that of color differentiation of Negribodies in
impression smears of specimen. Paraffin embedded sections of brain were stained by various methods. Schleifstein (1937) used Seller's method by rapid dioxan embedding. In USA a method described by Stoval and Black (1940) was used. In India Gangulee and Gangulee (1944) evolved a method in their Laboratory, which was based on colour differentiation of red and blue.

The third and the last stage in rabies diagnosis is characterised by specific identification of rabies antigen by specific immunologic techniques, immunofluorescence (Goldwasser and Kissling, 1958), immunoperoxidase (Levaditi et al., 1971) and ferritin tagged antibody method (Breese and Hsu, 1971) for electron microscopy.

The method adopted by Massignani and Malferrari (1961) for staining Negribodies employ eosin combined with phosphotungstic acid. With this method, Negribodies are stained deep red and Nuclei light blue. Thus the colour differentiation is very good. Therefore this method was selected for the purpose of study, along with a direct fluorescent antibody test which is now a well established diagnostic test for rabies. Immunoperoxidase technique, which is a new technique employing horse raddish peroxidase for the identification of rabies antigen in tissue cells was compared with these methods. In staining impression smears for giving rapid diagnosis of rabies, Sellers' stain, direct FAT and direct immunoperoxidase technique were applied and their efficiency in diagnosis was compared.
Passive cutaneous anaphylaxis reaction (PCA) in diagnosis of rabies as described by Mathew and Rao (1973) was studied for its routine applicability in diagnosis of rabies.

A request was sent to all the veterinary institutions to send brain materials from animals dying from rabies. Totally 14 dogs brains, two dog salivary glands, one buffalo brain, one cow brain and one horse brain were available during a 12 month period. These samples were inoculated in mice and a positive mouse inoculation test was obtained. LD$_{50}$ of these samples was calculated and 14 of these samples with varying LD$_{50}$ were selected for study. These 14 samples were inoculated in mice and after death of mice the impression smears of brain were examined by Sellers stain, direct FAT and direct IP. The results of these three methods were compared for efficiency. Sellers' stain detected Negri-bodies in 76.28 per cent of cases, where as direct FAT and direct IP detected rabies antigen in 92.5 per cent and 91.36 per cent cases respectively.

Thus it is observed that Sellers' technique is definitely less efficient in detecting Negri-bodies in impression smears as compared to direct FAT and direct IP. Smit (1963) reported efficiency of Negribody test 66 per cent.

Schneider and Wachendorfer (1964) reported 91 Negri-positive results in 224 brains as compared to 111 by FAT. Neuman (1965) reported 58.3 per cent positive by Seller's
stain. Hartwick and Shouman (1967) observed more doubtful cases of rabies by staining techniques than mouse inoculation. Subramanyam and Pathak (1971) detected Negribodies in only 15, whereas 23 samples were found positive by FAT (65.22 per cent). Salido and Romero (1967) found 64 per cent rabies positive by Seller's stain.

Stone et al. (1965) found FA and mouse inoculation more accurate than histological examination. Schneider (1964) considered FAT superior in accuracy and comparative rapidity, and Jentzsch (1965) stated advantages of FAT, speed, reliability and the fact that it can be used on decomposing tissue. Hüter (1966) reported that FAT yielded best results. Jentzsch (1967) demonstrated 98 per cent efficiency of FAT as compared to mouse inoculation, whereas Roslyakov et al. (1970) reported FAT as highly specific and more sensitive than mouse inoculation. Veeraraghavan et al. (1971) also reported FAT as more efficient as compared to other staining technique. Germano (1976) found direct FAT and mouse inoculation in best agreement with each other.

Levaditi et al. (1971) reported that when a purified antibody conjugated with peroxidase is allowed to react with fixed specimens, rabies antigen is clearly visible under high power of ordinary microscope. Various modifications of this method like FAT have been described (Mason et al., 1969, Sternberger et al., 1970). Immunoperoxidase test in diagnosis of rabies is satisfactory in case of brains with a high concentration of virus but is not dependable when virus
concentrations are low (ICMR, 1977). Taylor (1978) listed the disadvantages of the fluorescent antibody technique like need of a specialised microscopic equipment, impermanancy of stained slides, nonspecific reactions etc. These disadvantages are largely avoidable by immunoperoxidase technique. The present advantages of FAT are that it is less time consuming and wide range of FITC conjugates are available commercially. Genevose and Andral (1978) observed that immunoperoxidase test remains a useful and sufficiently sensitive diagnostic method particularly in laboratories not able to carry out fluorescent microscopy.

In our studies results obtained by examining impression smears compares well with literature reported. However, the relative positive figures are higher than Neuman (1965), Subramanyam and Pathak (1971) and Salido and Romero (1967) have reported in case of Sellers stain but the results of FAT are equally good, showing 92.5 per cent positive samples. Direct immunoperoxidase technique is found equally sensitive to FAT showing 91.36 per cent positive samples.

In case of paraffin embedded sections, all the three methods tested, are of equal efficiency and therefore were found equally sensitive. Bosc (1966) has recommended Massegnani and Malferri (1961) method for staining Negri bodies in paraffin sections. This method uses the stains and reagents usually found in a laboratory and readily available, and high power ordinary light microscope is required like immunoperoxidase technique. Thus in case of
paraffin embedded sections all the three methods/tests are equally sensitive, as reported in the literature, but in case of impression smears for giving rapid diagnosis of rabies, FAT appears most sensitive, which can be replaced by immunoperoxidase technique where the facility of fluorescent microscopic equipment is not available. Horse radish peroxidase Type IV RZ 3.0, sigma USA was found most suitable for the test.

All these tests were performed with the brains containing LD$_{50}$ ranging from $10^{-2.15}$ to $10^{-5.85}$ and there was no indication that samples containing low concentration of virus would have given negative results.

The most reliable test in diagnosis of rabies is mouse inoculation which also at times gives negative result and more over it will take 21 days for confirmation. FAT is equally reliable test but needs a fluorescent microscope, and an experienced worker.

The passive cutaneous anaphylaxis reaction is a comparatively quick and simple test which takes about three to four hours. This test is used as tool in various immunological studies involving the skin sensitizing antibodies. Mathew and Rao (1973) applied this test in diagnosis of rabies.

Guinea pigs were chosen for the experiment as they are the best suited experimental animals. Mice are useful but there was a high rate of mortality probably due to embolism occurring by intravenous injection of antigen and dye. Antirabies serum from guinea pig, rabbit, horse and dog
were used and the rabies positive brains were used as a source of antigen. The positive brains used in the study of comparison of staining techniques, FAT and IP were only used in this study. First the PCA reaction was studied by using CVS antigens from IVRI and CRI- Kasauli. The horse serum did not give good results, neither guinea pigs gave better results. Better results were obtained by rabbit and dog antirabies sera.

Mathew and Rao (1973) also reported the same results. Horse antirabies serum was found unsuitable, and same was the case with regard to guinea pig serum. However for further study with street virus antigens, horse serum was dropped but antirabies sera from guinea pig, rabbit and dog were taken.

With five street virus antigens and two CVS antigens with LD$_{50}$ ranging from $10^{-2.15}$ to $10^{-5.16}$, guinea pig antirabies serum gave PCA reaction of four mm in diameter. With normal serum from guinea pigs doubtful reactions were observed in two cases and in one case normal serum gave a positive result. However, when the same serum was tested in a dilution of 1:2, 1:4, 1:8, 1:16 and 1:32 with seven antigens, all antigens gave a reaction of bluing of four mm in diameter. There was no difference in the reaction at serum dilution of 1:2 and in 1:4 dilution two antigens were negative.
The PCA reaction in guinea pigs with guinea pig anti-rabies serum did not produce satisfactory results leading one to believe its specificity beyond doubt. Same results are reported by Mathew and Rao (1973).

In case of antirabies serum from rabbits, all the seven antigens gave good results showing bluing of 12 mm diameter. In serial dilutions of 1:2, 1:4, 1:8 and 1:16 also the reaction was of same level as that of whole serum except in case of two antigens having \( \text{LD}_{50} 10^{-2.15} \) and \( 10^{-2.50} \). These were the antigens having lowest \( \text{LD}_{50} \) amongst seven antigen tested. However it cannot be said with certainty that one degree mild reaction was due to \( \text{LD}_{50} \) as the number of samples tested are small. At 1:4 dilution too, the PCA reaction was very detectable with a bluing of eight mm diameter. Intense reaction in case of CVS antigens was observed upto 1:4 dilution and bluing upto four mm diameter was observed at a dilution of 1:16. Incidentally these two antigens contained highest \( \text{LD}_{50} \).

Antirabies serum from dog when tested with seven antigens showed equally good results comparable to those obtained in case of rabbit sera. In this case too, CVS antigens gave intense reaction at 1:2 and 1:4 dilution, and bluing upto four mm in diameter was observed at 1:16 and 1:32 dilutions.

Thus it can be concluded that out of the antirabies sera obtained from horse, guinea pig, rabbit and dog when tested with rabies antigen, rabbit and dog
sera gave satisfactory results, even at a dilution of 1:2 there is no difference observed in intensity of reaction.

All these street virus strains were inoculated in mice and were found positive by mouse inoculation test. The brains harvested from these mice were tested for presence of rabies antigen by FAT, direct IF and by histologic staining techniques and were found positive. Therefore it can be concluded that PCA reaction can be used as diagnostic test in rabies. However studies involving large number of field samples are necessary for determining its sensitivity and specificity beyond doubt.