1. Gunshor, R. and Nuvorikko, A., *Sci. Am.*, July 1996.
2. Isamu Akasaki, Hiroshi Amano, Shigetoshi Sota, Hiromitsu Sakai, Toshiyuki Tanaka and Masajoshi Koike, *Jpn. J. Appl. Phys.*, 1995, **34**, L1517.
3. Nakamura, S., Senoh, M., Nagahama, S. *et al.*, *Jpn. J. Appl. Phys.*, 1996, **35**, L174.
4. Nakamura, S., Senoh, M., Nagahama, S. *et al.*, *Jpn. J. Appl. Phys.*, 1996, **35**, L217.
5. Nakamura, S., Senoh, M., Nagahama, S. *et al.*, *Appl. Phys. Lett.*, 1996, **68**, 2105.
6. Robert F. Davis, *Proc. IEEE*, 1991, **79**, 702.
7. Paul, T. K., Bhattacharya, P. and Bose, D. N., *Electron. Lett.*,1989, **25**, 1602.
8. Paul, T. K., Bhattacharya, P. and Bose, D. N., *Appl. Phys. Lett.*, 1990, **56**, 2648.
9. Ghosh, S., Ph D thesis, Materials Science Centre, Indian Institute of Technology, Kharagpur, 1996, p. 111.
10. Kumar, A., Pal, D. and Bose, D. N., *J. Electron. Mat.*, 1995, **24**, 833.
11. Dissanayake, A., Lin, J. Y., Jiang, H. X., Yu, Z. J and Edgar, J. H., *Appl. Phys. Lett.*, 1994, **65**, 2317.
12. James H. Edgar (ed.), *Properties of Group III Nitrides*, INSPEC, IEE, London, UK, 1994, p. 232.

ACKNOWLEDGEMENT. We are grateful to the National Laser Programme for a project on 'Semiconductor Lasers' under which this work was carried out.

Received 19 December 1996; revised accepted 17 March 1997

Detection of the *lcr* gene in *Yersinia pestis* responsible for the recent outbreak of plague in India

S. S. Lahiri and B. S. Karothia

Defence R&D Establishment, Jhansi Road, Gwalior 474 002, India

Rapid detection of plague bacilli is particularly germane to plague epidemiology since untreated bubonic plague can rapidly progress to septicaemic or pneumonic state. A 1.3 kb *Hind*-III/*Xho*-I DNA fragment from the plasmid pCd-1 which codes for the low Ca^{2+} response of *Yersiniae* spp. has been used to screen the isolates of *Y. pestis* obtained from the recent outbreak at Beed and Surat. The probe discriminated *Y. pseudotuberculosis*, diarrhoeagenic *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Shigella dysenteria* from *Y. pestis* and *Y. enterocolitica*. Both radioactive and non-radioactive probes gave excellent result.

THE once dreaded bacteria, *Y. pestis* is known to carry three plasmids of 9.5 kb, 70 kb and 95 kb size[1]. While the 9.5 kb (pPst) and 95 kb (pFra) plasmids code for the pesticine and F1 antigen respectively, the 70 kb (pCad) plasmid is responsible for the low calcium response (LCR). Expression of virulence (V-antigen) is controlled by the *lcr* gene[2].

Virulent *Yersiniae* spp. can be detected by the presence of the LCR plasmid which, besides conferring virulence to the microorganism, makes the bacteria dependent on Ca^{2+} for growth at 37°C, expression of novel outer membrane protein called *Yersinia* outer protein (Yop), increased hydrophobicity, increased affinity to crystal violet and congo red, a tendency to agglutinate in media containing mammalian serum or enhanced resistance to bactericidal effects of normal human serum[3]. Although these parameters can be used for *in vitro* detection of the microorganism, they are reported to be not reliable tests[3]. Laboratory screening requires animal passages to isolate a pure culture, phage sensitivity typing, immunofluorescent antibody against fraction-1 (a major capsular antigen), etc. Screening by these methods is expensive and time-consuming. Detection at the nucleic acid level circumvents all the above limitations and provides a rapid and sensitive method for its specific detection.

In the present study, an attempt has been made to detect the presence of *lcr* gene in *Y. pestis* by DNA probe in different human and rodent isolates obtained during the recent outbreak at Beed and Surat districts. The other two virulent non-pestis *Yersiniae*, which include *Y. pseudotuberculosis* and *Y. enterocolitica* and other diarrhoeagenic enterobacteria like *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella dysenteriae* and *Salmonella typhi* were also tested both by radioactive and non-radioactive DNA probes.

The test samples used comprised of 11 clinical isolates of *Y. pestis* from pneumonic patients of Beed district and Surat city: seven isolates from rodents, *Y. pestis* strain no. A-1122 which do not carry the LCR plasmid; *Y. pseudotuberculosis* strain no. 1A which was kindly supplied by May C. Chu, WHO Collaborating Centre at Centre for Disease Control, Fort Collins, USA; the *Y. enterocolitica* strain no. 0:9 IP 383 was kindly provided by Elizabeth Carniel, WHO Collaborating Centre, Institute Pasteur, Paris. Samples of *E. coli*, *K. pneumoniae*, *S. dysenteriae* and *S. typhi*, available in DRDE, were also tested simultaneously.

Bacterial cultures (24 nos) were scraped from agar plates and each resuspended in 100 µl of 0.5 M Tris.HCl (pH 8.0), 0.1 M sodium chloride and 0.01 M ethylene diamine tetraacetic acid (EDTA) in different eppendorf tubes and lysed by lysozyme followed by heat treatment.

The 1.3 kb restriction fragment that has been used as the DNA probe, contained the 3′ end of *lcr-R*, the complete *lcr-G* and part of the *lcr-V* (virulence antigen) gene of the 70 kb LCR plasmid pCD-1. *E. coli* with the recombinant plasmid vector pES6-1, which contains the above as well as the *lcr-H*, *yop-B* and *yop-D* genes

of pCD-1 was obtained from S. C. Straley, University of Kentucky, USA. A 1.3 kb *Hin*d-III/*Xho*-I restricted DNA fragment from pES 6-1 was used as the DNA probe.

This DNA fragment was radiolabelled with α^{32}P deoxycytidine triphosphate (dCTP) obtained from Board of Radiation and Isotope Technology, Bombay, with the help of the Klenow fragment of DNA polymerase-I (M/s Bangalore Genie Pvt Ltd) and random hexanucleotide primer (M/s Boeringer Mannheim, Germany). Non-radioactive labelling of the 1.3 kb DNA fragment was performed with digoxigenin. Both the methods of labelling were performed as per standard protocols[4].

Samples (5 µl each) were spotted on nitrocellulose membrane. A total of 24 samples were spotted in four rows and the filter was processed through the steps of denaturation, neutralization and baking.

Prehybridization and hybridization (16 h) were performed at 65°C in 6 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate pH 7.0), 5 × Denhardt's solution (1 × is 0.02% each of Ficoll, polyvinyl pyrrolidone and bovine serum albumin), 0.02 M phosphate buffer, 0.5% SDS and 100 µg/ml of heat denatured sonicated Salmon sperm DNA. Post hybridization washing comprised of washing thrice in 2 × SSC and 0.5% SDS for 10 min each at room temperature followed by in 1 × SSC and 0.1% SDS for 30 min at 55°C and in 0.5 × SSC and 0.1% SDS for 30 min at 65°C.

The infrared dried filter was put for autoradiography overnight at −20°C in case of radioactive detection. In case of non-radioactive detection, the filter was processed as advised by the manufacturer and detected by antidigoxigenin antibody conjugated with alkaline phosphatase.

Initial screening was performed with radioactive probe for 10 samples which included 3 *Y. pestis* isolated till then (isolate nos. 1, 2 and 3), *S. dysenteriae*, two isolates each of *E. coli*, *K. pneumoniae* and *S. typhi*. The 1.3 kb DNA probe detected the three *Y. pestis* (Figure 1) specifically.

Figure 2 shows the result of non-radioactive detection wherein all the 18 *Y. pestis* isolates were detected. The *Y. pestis* strain no. A-1122 which is deprived of the pCad plasmid was not detected. Although the *Y. enterocolitica* (sample no. 21) was detected as positive, the *Y. pseudotuberculosis* (sample no. 20) was not detected in repeated experimentation. Similarly *E. coli*, *K. pneumonia* and *S. typhi* (sample nos. 22, 23 and 24 respectively) were not detected.

Radioactive DNA probe used to screen all the 24 samples (Figure 3), used for further confirmation of results of non-radioactive detection, detected all the isolates which were detected by the non-radioactive DNA probe.

The samples detected as positive by the *lcr* probe has been earlier confirmed[5] to be *Y. pestis* by different classical, micro-biological, biochemical, fluorescent antibody staining and specific phage infection.

The virulence-associated low calcium response plasmid (pCd-1 also called as pCad) is shared by all the virulent spp. of *Yersinia*, viz. *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. The LCR brings about coordinate regulation of plasmid encoded virulence genes by environmental temperature and calcium[2]. The *lcr* gene has been studied by several authors in the recent past[2–9].

The LCR plasmid varies between 70 kb (refs 10, 11) and 75 kb (ref. 6) in various spp. and strains and the Ca^{2+} dependence region spans approximately 25 kb portion[6] of the LCR plasmid. This portion of the plasmid regulates expression and secretion of anti-host proteins like LCR-V (virulence protein which is a protective antigen) and about 10 Yops. It is a regulatory response in which a set of virulence-related operons are strongly induced at 37°C in the absence of Ca^{2+} and partially down regulated at 37°C when Ca^{2+} is present in mM concentrations[5].

Figure 1. Autoradiograph showing three *Y. pestis* isolates harboring the LCR plasmid. The *E. coli*, *K. pneumoniae*, *S. dysenteriae* and *S. typhi* were not detected.

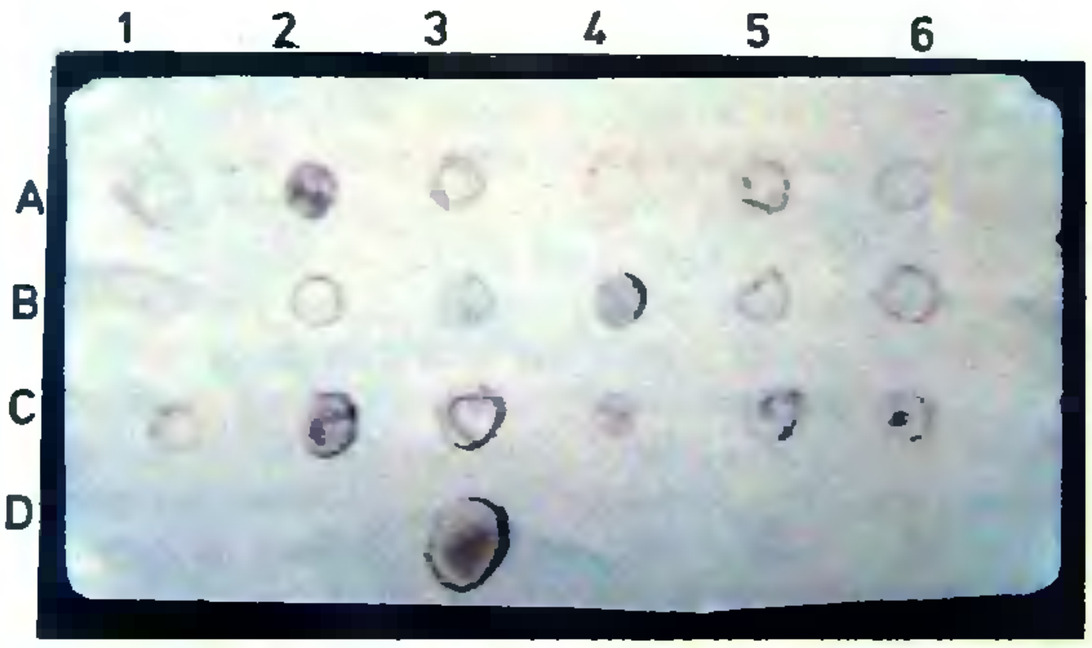

Figure 2. Digoxigenin labelled DNA probe detects all the *Y. pestis* isolates and *Y. enterocolitica* which carry the LCR plasmid. Sample nos A1 to B5 are the human isolates of *Y. pestis*, B6 to C6 are rodent isolates, D1 is *Y. pestis* A-1122, D2 is *Y. pseudotuberculosis*, D3 is *Y. enterocolitica*, D4, D5 and D6 are *E. coli*, *K. pneumoniae* and *S. typhi* respectively.

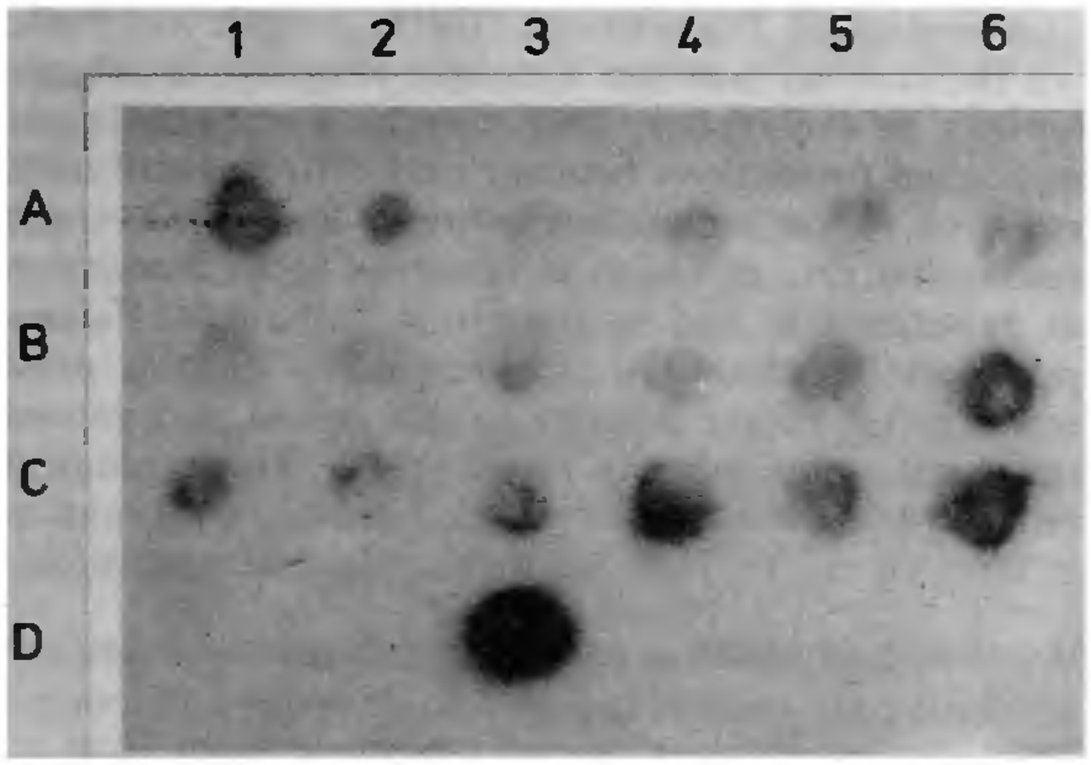

Figure 3. Radiolabelled DNA probe detects all the *Y. pestis* isolates and *Y. enterocolitica* which carry the LCR plasmid. Sample nos A1 to A6 and B2 to B6 are the human isolates of *Y. pestis*, B1 and C1 to C6 are rodent isolates, D1, D2 and D3 are *Y. pestis* A-1122, *Y. pseudotuberculosis* and *Y. enterocolitica* respectively, D4, D5 and D6 are *E. coli*, *K. pneumoniae* and *S. typhi* respectively.

All the *Y. pestis* isolates have been specifically detected by the 1.3 kb probe, excepting the strain no. A-1122 which is deprived of the LCR plasmid. Thus all the isolates of *Y. pestis* obtained from the clinical samples during the recent outbreak are harbouring the 70 kb LCR plasmid. This has also been confirmed earlier by pulse field gel electrophoresis[12]. The virulent *Y. enterocolitica*, as expected, has also been found to carry the LCR plasmid. Both the radioactive and non-radioactive DNA probes gave identical results, confirming the faint spots revealed in the non-radioactive detection, as distinct positives.

The *Y. pseudotuberculosis*, however, was not detected by the probe even on repeated experimentation. We have reduced the stringencies confining post-hybridization washings to 55°C with 0.5× SSC and 0.1% SDS but even so there was no hybridization with the *Y. pseudotuberculosis*. The possible explanation for non-detection of *Y. pseudotuberculosis* could be (i) due to the loss or curing of the plasmid which is a common feature of the dynamic plasmid. In their studies with the 67 kb yVA-2635 plasmid of *Y. enterocolitica*, Robins-Browne[3] observed that this is a low-copy plasmid which has a high rate of spontaneous curing. They have also observed that none of the avirulent strains hybridized with the probe although they harbored the 50–70 kb plasmid. Two of the isolates detected as positive by the virulence gene probe had substantial deletion in the plasmid but still retained their virulence. They also observed that all the *Yersinia* strains carrying the LCR plasmid were virulent and that 11 of the plasmid cured strains lost their virulence. The authors therefore concluded that DNA probes consisting of the LCR-related virulence gene is an ideal test method for specific and sensitive detection of virulence of *Yersinia*. (ii) It has been reported[3] that although substantial length of the virulence plasmid is highly conserved, there exist regions of low degree homology in different spp. and strains of *Yersiniae*. It is possible that the 1.3 kb DNA fragment of the pCd-1 plasmid that we have used as the probe could be absent in the *Y. pseudotuberculosis* strain studied. Further studies with large number of strains will clarify this point.

Different other assay systems for testing virulence associated with the LCR plasmid of *Yersiniae* include Ca^{2+} dependence (which is difficult to perform, besides that it is least sensitive and specific a test of plasmid carriage), pyrazinamidase activity (also difficult to perform), crystal violet assay (a relatively better assay although it gave few false positive detections)[3]. Detection of *Y. pestis* by conventional method, besides being expensive, may take as long as two weeks' time[10]. Earlier studies by McDonough *et al.*[11] with immunodetectable antibody against the F1 antigen have proved unreliable. Chromosomal probes like invasion (inv) and attachment invasion locus (ail) have been reported to be good genus-specific probes but they cannot detect virulence *per se*[3]. The Ca^{2+} dependence region on the other hand, is a highly conserved sequence[3] and good indicator of virulence.

Detection of *Y. pestis* by polymerase chain reaction with primer pairs p1 and p2 for the 9.5 kb pPst plasmid, C1 and C2 primers for the 70 kb pCad plasmid and F1 and F2 for the 95 kb pFra plasmid have been reported[1] as an improved method. Similarly polymerase chain reaction for detection of the 501 bp *caf-1* gene fragment and a 443 bp of *pla* gene fragment from the 100 kb pFra and 10 kb pPst plasmid respectively has been reported[13] to be of useful diagnostic value.

Although recent developments including detection of *Y. pestis* through its F1 antigen with the help of a fibre optic biosensor[14] for rapid (few minutes) detection and other improved methods like polymerase chain reaction and ribotyping are available, detection of virulent *Y. pestis* by DNA probe is most promising for its cost-effectiveness, specificity, sensitivity and ability to screen a large number of samples simultaneously and inexpensively. Detection of *Y. pestis* (by a modified colony hybridization method) directly from the rat-flea[10] which transmits the plague bacilli among rats and from rats to human has been reported. The 900 bp DNA probe isolated from the 9.5 kb pKyp-1 plasmid has been reported to be suitable for plague surveillance in fleas. The *lcr* gene probe shall have to be tried for direct detection of *Y. pestis* from clinical samples as well as from infected animal tissues and carcasses. For this purpose, detection with non-radioactive system involving digoxigenin and antidigoxigenin is convenient for rapid

detection in even small laboratories as rapid detection of plague bacilli is particularly germane to plague epidemiology since untreated bubonic plague rapidly progress to septicaemic or pneumonic state.

1. Kulichenko, A. N., Narkina, O. V., Gintsburg, A. L., Popov, Iu. A. and Ddrozodov, I. G., *Genetika*, 1994, 30, 167–171.
2. Fields, K. A., Plano, G. V. and Straley, S. C., *J. Bacteriol.*, 1994, **176**, 569–579.
3. Robins-Browne, R. M., Miliotis, M. D., Cianciosis, S., Miller, V. C., Falcow, S. and Morris, I. G. Jr., *J. Clin. Microbiol.*, 1989, **27**, 644–650.
4. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, USA, 1989.
5. Batra, H. V., Tuteja, U. and Agarwal, G. S., *Curr. Sci.*, 1996, **71**, 787–791.
6. Straley, S. C., Plano, G. V., Skrzypek, E., Haddix, P. L. and Fields, K. A., *Mol. Microbiol.*, 1993, **8**, 1005–1010.
7. Skrzypek, E. and Straley, S. C., *J. Bacteriol.*, 1995, **177**, 2530–2542.
8. Straley, S. C. and Perry, R. D., *Trends Microbiol.*, 1995, **3**, 310–317.
9. Plano, G. V. and Straley, S. C., *J. Bacteriol.*, 1995, **177**, 3843–3854.
10. Starley, S. C., Skrzypek, E., Plano, G. V. and Bliska, J. B., *Infect Immun.*, 1993, **61**, 3105–3110.
11. McDonough, K. A., Schwan, T. G., Thomas, R. E. and Falkow, S. J., *Clin. Microbiol.*, 1988, **26**, 2515–2519.
12. Panda, S. K., Nanda, S. K., Ghosh, A. *et al.*, *Curr. Sci.*, 1996, **71**, 794–799.
13. Norkina, O. V., Kulichenko, A. N., Gintsburg, A. L., Tuchkov, I. V., Popov, Yu. A., Aksenov, M. V. and Drosdov, I. G., *J. Appl. Bacteriol.*, 1994, **76**, 240–245.
14. Cao, L. K., Anderson, G. P., Ligler, F. S. and Ezzell, J., *J. Clin. Microbiol.*, 1995, **33**, 336–341.

ACKNOWLEDGEMENTS. We thank Dr R. V. Swamy for encouragement and support. We also thank the Chairman and members of the Technical Advisory Committee, Govt of India, for giving us an opportunity for undertaking this work. Thanks are also due to Dr H. V. Batra and Mrs U. Tuteja for supplying the test samples.

Received 13 January 1997; revised accepted 20 March 1997

Correlation of alpha-logger radon data with microseismicity in N-W Himalaya

H. S. Virk, Anand K. Sharma and Vivek Walia

Department of Physics, Guru Nanak Dev University, Amritsar 143 005, India

Six alpha-logger stations have been set up in the Kangra and Chamba Valleys of Himachal Pradesh (India) in N-W Himalaya to monitor radon emanation continuously. Time series radon data is recorded at all the stations from March–April 1993 to August 1995. A number of impulsive radon spikes are recorded at all the stations, most of which are correlatable with seismic activity reported by the India Meteorological Department (IMD) seismic network. All the stations manifest a unique response to seismic activity in the region. This may be due to different geological formations beneath and different diffusion rates of radon emanation at each station. Average emanation rate of radon is observed to be maximum at Jawalamukhi and minimum at Pathankot. Palampur and Jawalamukhi are found to be the most sensitive to seismic activity in the region and record the maximum number of radon spikes. Meteorological analysis of radon data revealed that radon emanation is affected up to 15% by meteorological variations.

RADON is established as a useful geochemical precursor[1-9] in earthquake prediction research since the observation of increase of anomalous radon in a deep well in Tashkent before the 1966 Tashkent earthquake[10]. The physical bases of the radon anomalies, prior to an impending earthquake, have yet to be fully understood in terms of a comprehensive theoretical model. However, it is understood that the buildup of stresses prior to a seismic event can alter the radon concentration at a position where such premonitory changes can be measured. If the earth's deformation, prior to an earthquake, releases increased amounts of radon, the temporal increase at the surface of the earth can be used to anticipate seismic activity.

The northern boundary of the Indian sub-continent, extending from the Hindukush in the west to the hills of Assam and Burma in the east, constitutes a region where the Indian plate collides with the Eurasian plate and has thus been the scene of a large number of major earthquakes in the past. During the last two decades, the N-W Himalaya has recorded five earthquakes of magnitude more than 5, viz. Kinnaur (19 January 1975), Dharamshala (14 June 1978), Dharchula–Bajang (29 July 1980), Jammu–Kathua (14 August 1980) and Dharamshala (26 April 1986). The Hindukush area and the Kangra valley of Himachal Pradesh (India) are considered to be highly seismic zones in the N-W Himalayan belt which is traversed by several major thrust faults.

Radon monitoring work was started in 1989 at Palampur in Kangra valley using the track-etch technique and emanometry. The results of the studies are reported elsewhere[8-9,11]. Under Himalayan Seismicity Project of the Department of Science and Technology (DST), Government of India, six alpha-logger probes were installed at Palampur (32.10°N, 76.51°E), Pathankot (32.30°N, 75.64°E), Kotla (32.35°N, 76.02°E), Dalhausie (32.60°N, 76.00°E), Chamba (32.55°N, 76.10°E) and Jawalamukhi (31.87°N, 76.33°E) (Figure 1). Some of these stations are situated in close proximity to the main boundary thrust (MBT) of the Himalaya. The Kangra and Chamba valleys in Himachal Pradesh are enclosed between the middle Siwaliks and the Dhauladhar range and the Pir Panjal and the Dhauladhar ranges of lesser