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Genetic Fingerprinting of *Bacillus thuringiensis* Isolates by Randomly Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

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Abstract

Random Amplified Polymorphic DNA (RAPD) is a method of producing a genetic fingerprint of a particular species without its prior genetic information. Relationship between species may be determined by comparing their unique fingerprint information. *B. thuringiensis* was isolated from soil samples of Khumbu base camp of Everest region, Nepal. Crystal protein (delta endotoxin) producing strains (46 from Phereche and 40 from Sagarmatha national park) were tested against a series of 100 decamer RAPD primers (codes 201-300, obtained from University of British Columbia) by RAPD PCR. Primer 284 was found the best among the tested primers and the reaction condition for PCR was optimized with a PCR buffer containing 10mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$ with pH 8.3.; 200ìm dNTPs each, 1U Taq polymerase , 40 pmol decamer primers, 20 ng template DNA and 1% DMSO as a final concentrations in 25ìl reaction mixture. The thermal programme was programmed as initial denaturation temperature at 94°C for 5 min followed by 35 cycles with denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 mins with final extension temperature at 72°C for 10 min. Higher polymorphic fragments were found in the range between 700-900 bp. Next to it, the range of 400-700 and 1200-1600 bp were, too, highly polymorphic among the isolates. The discriminatory capacity (D) of the RAPD-PCR was found to be 0.9901. The isolates of cold tolerant *B. thuringiensis* from high altitude regions were found rich in genomic polymorphism.

Key words: RAPD-PCR, fingerprint, endotoxin, *Bacillus thuringiensis* Berliner, polymorphism, base pair (bp)

Introduction

Use of chemical pesticides has led to the emergence and spread of resistance in agricultural pests and vectors of human diseases and to the environmental degradation. The very properties that made these chemicals useful-long residual action and toxicity to a wide spectrum of organisms-have brought about serious problems. An urgent need has thus emerged for environment friendly pesticides to reduce contamination and the likelihood of insect resistance (Ben-Dov *et al*. 1997).

The soil bacterium *Bacillus thuringiensis* Berliner fulfills the requisites of a microbiological control agent against agricultural pests and vectors of diseases that lead to its widespread commercial application. It is a gram-positive, aerobic, endospore-forming saprophyte. All known subspecies of *B. thuringiensis* produce large quantities of insecticidal crystal proteins (Cry proteins) which are segregated in parasporal bodies (also known as ä-endotoxins). The genes coding for Cry proteins normally occur on large plasmids and direct the synthesis of a family of related Cry proteins classified as Cry1-28 and Cyt1-2 groups according to their degree of amino acid homology. Cry proteins have been used as biopesticide sprays on a significant scale for more than 30 years, and their safety has been demonstrated. The main target pest of *B. thuringiensis* include various lepidopterous (butterflies and moths), dipterous (flies and mosquitoes), and coleopterous (Beetles) species. Some strains have also been found

to kill nematodes (Schenpf *et al*. 1998). Conventional *B. thuringiensis* preparations such as those registered in Germany and found worldwide are mostly derived from the highly potent strain *B. thuringiensis* var. *kurstaki* HD1, which was isolated in the sixties (Dulmage 1970).

Williams *et al*. (1990) used varieties of morphological and physiological characteristics to assign different bacterial strains into defined taxonomical clusters. However, most of the taxonomic methods are very time consuming and sometimes give ambiguous results. Genomic fingerprinting assays using RAPD have already been shown to be useful for differentiation of bacterial strains. This method is based on the amplification of distinct DNA sequences under low stringency conditions during annealing using an oligonucleotide of arbitrary sequence. The primer is not directed at any specific sequences within the template, making previous knowledge of the genome non-essential. The efficacy of the amplification procedure is primarily dependent on sufficient sequence similarity at the 3' end of the oligonucleotide to allow adequate priming. The resulting pattern of amplification products of varying size can subsequently be used as a genetic fingerprinting of the organisms (Mehling *et al*. 1995) and can also be used to genetically link to a trait of interest for individual and pedigree identification, pathogenic diagnostics, and trait improvement in genetics and breeding programmes. Morphological , biochemical characterization and identification, isozyme analysis, restriction fragment length polymorphism (RFLP), minisatellites, microsatellites , randomly amplified polymorphic DNAs (RAPD) and fluorescence *in situ* hybridization (FISH) have been so far used to analyse genetic similarity and diversity for breeding research of animal/plant/microbes (Yoon & Kim 2001). In this study RAPD-PCR has been used for genetic and molecular studies as it is a simple and rapid method for determining genetic diversity and similarity in various organisms.

Materials and Methods

Bacterial isolates

B. thuringiensis were isolated by acetate selection method from the soil samples collected from Khumbu base camp of Everest region. The isolates were identified by standard microbiological techniques including colonial, morphological and biochemical characteristics according to Bergey's manual of systematic bacteriology (Claus & Berkeley 1986).

Preparation of template DNA

Templates were prepared from 16 to 18hr cultures in Luria-Bertani medium as described by Ben-dov *et al.* (1997). Aliquots of 3 to 4.5 ml were harvested by centrifugation and washed once in TES (10 mM Tris-HCl of pH 8.0, 1 mM EDTA, 100 mM NaCl), and the pellets were resuspended in 100 ml of lysis buffer (25% sucrose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 4 mg of lysozyme per ml). The cell suspension was incubated for 1 hr at 37°C. Further, DNA extraction was performed as described by Sambrook *et al.* (1989). Extracted DNAs were quantitated by spectrofluorometer and diluted up to 20ng DNA/ul in order to feed on the PCR reaction mixture.

RAPD reaction

One hundred RAPD primers (10-mers) of arbitrary sequence obtained from University of British Columbia, Canada, were screened for the ability to produce discriminatory polymorphisms. RAPD-PCR mixture was set up that contained 20-50 ng of genomic DNA, 40 pmol of primer, 1 U of Taq polymerase (Bangalore GENEI.), 200 uM (each) deoxynucleoside triphosphate, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 3 mM MgCl2 and 1% DMSO. Each reaction mixture was overlaid with 25 ul of mineral oil and amplified with a Perkin-Elmer Cetus DNA Thermal Cycler model TC-1 as follows: (i) Initial denaturation, 1 cycle consisting of 5 min at 94°C and (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 2 min at 36°C, and 3 min at 72°C, followed by a final extension step at 72°C for 10 min.

RAPD products were separated by agarose gel electrophoresis (1 %) with 1X TAE buffer for 2 hrs. Molecular size standards (λ DNA *Hin*dIII digest and ϕ X 174 phage DNA type II digest) were also included in each gel, photographed by the Fototdyne camera using polaroid film (Porplan 667). The RAPD fingerprints were analyzed visually and the molecular size of each band migrated was calculated by plotting standard curve (log of molecular weight vs distance travelled) of the standard DNA ladders.

Results

Ninety one soil samples collected from the Khumbu base camp of Everest region, Nepal were processed at Research Laboratory for Biotechnology and Biochemistry. A total 109 *B. thuringiensis* isolates

were obtained from the soil samples of Phereche (P) and Sagarmatha national park (SNP). From 52 Phereche soil samples, 63 isolates were obtained and from 39 soil samples from SNP, 46 isolates were obtained but only 86 isolates were found to produce crystal protein which were preceded for RAPD-PCR (Shrestha *et al.* 2006)

Identification of discriminatory primer(s) for RAPD analysis

Nine primers (Table 1) with GC content - 50-80%, were found to amplify genomic DNA fragments with reproducible polymorphisms suitable for strain differentiation of the *B. thuringiensis* isolates (Fig 1). Primer 284 with GC content 70% was found to produce more polymorphic bands than other primers and was used to obtain RAPD profiles of some selected *B. thuringiensis* isolates (Fig 2 and 3).

Table 1. RAPD primers producing reproducible polymorphisms with *B. thuringiensis*

S.No	Primer	Sequences of primers (5' to 3')	GC% of primers	No. of bands produced	Molecular size of the bands (bp)
1	208	ACG GCC GAC C	80	8	2821, 1842, 1473, 1282, 747, 639, 502, 357
2	254	CGC CCC CAT T	70	6	4228, 3102, 2357, 1125, 789, 639
3	256	TGC AGT CGA A	50	2	1125, 372
4	268	AGG CCG CTT A	60	6	2575, 1583, 1282, 936, 789, 404
5	275	CCG GGC AAG C	80	8	4228, 2575, 1282, 993, 789, 672, 526, 372.
6	276	AGG ATC AAG C	50	7	2357, 1473, 1282, 1056, 936, 747, 639
7	**284**	**CAG GCG CAC A**	**70**	**10**	**3425, 1995, 1583, 1373, 1200, 936, 834, 747, 639, 480**
8	292	AAA CAG CCC G	60	3	1373, 993, 708
9	299	TGT CAG CGG T	60	2	1373, 993

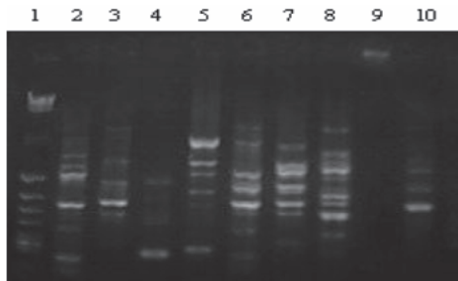

Fig 1: Screening of Primers producing reproducible polymorphisms.
Lane 1: DNA marker λ-DNA/HinIII digest,
Lane 2-10: primer 208, 254, 256, 268, 275, 276, 284, 292 and 299 respectively.

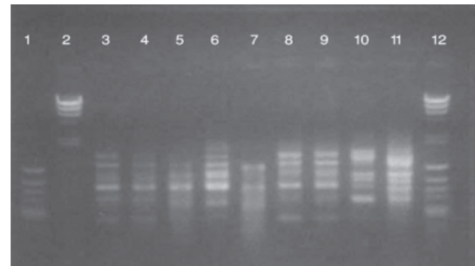

Fig 2: RAPD profiles of different *B. thuringiensis* isolates Lane 1, 2 &12-Molecular size marker (φ174 HaeIII cut, λ DNA HinIII cut Mixed molecular size markers. Lane 3-5, P_{34}, Lane 6-11, P_{37}, P_{38}, P_{39}, P_{39}, P_{42} and P_{43} respectively.

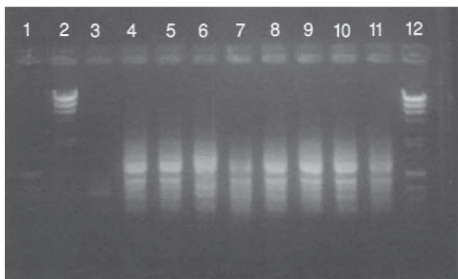

Fig 3: RAPD profiles of different *B. thuringiensis* isolates Lane 1, 2 &12-Molecular size marker (φ174 HaeIII cut, λ DNA HinIII cut Mixed molecular size markers. Lane 3-11, P_{44}, P_{45}, P_{46}, P_{48a}, P_{49}, P_{50}, P_{51b}, P_{52} and P_{53} respectively.

Of 108 isolates, 86, the crystal protein producers, were typed by RAPD-PCR to study the polymorphism patterns. The RAPD typing results are summarized in Table 2, 3 and 4.

RAPD fingerprinting of the *B. thuringiensis isolates*

Table 2. RAPD types of the Phereche isolates

S.No	Codes of theisolates	No. of RAPD bands produced	Number of the base pairs (bp)
1	P_1	1	2061
2	P_2	1	812
3	P_3	6	1843, 1563, 1213, 962, 690, 495.
4	P_5	4	1563, 1053, 811, 690
5	P_6	2	1274, 811
6	P_7	7	1378, 788, 738, 692, 611, 512
7	P_{9a}	10	2061, 1436, 1270, 1173, 1046, 937,815, 738, 692, 630
8	P_{10}	1	1563
9	P_{12a}	6	2139, 1944, 1629, 1501, 1337, 1200
10	P_{12b}	1	2497
11	P_{13}	6	1648, 1449, 1187, 1023, 889, 775
12	P_{14}	1	1499
13	P_{15}	5	3148, 2638, 1944, 1389, 927
14	P_{17b}, P_{25}, P_{27}, P_{28},P_{30}	2	1944, 1501
15	P_{18}	6	2368, 2139, 1775, 1629, 1501, 1200
16	P_{21}	1	1629
17	P_{22}	6	3353, 2638, 2139, 1050, 1289, 1121
18	P_{24}	2	2139,1700
19	P_{26}	2	2249, 1944
20	P_{29}	3	2638, 1944, 1501
21	P_{31}	3	1086, 670, 527
22	P_{32}	1	1501
23	P_{33}	4	1156, 921, 811, 551.
24	P_{34}	5	1553, 1326, 763, 567, 375
25	P_{35}	10	1499, 1378, 1128, 972, 873, 788, 738, 692, 542, 512
26	P_{37}	7	2006, 1553, 1229, 991, 717, 508, 413
27	P_{38}	3	1142, 717, 567
28	P_{39}	6	1553, 1229, 991, 763, 600, 375
29	P_{40}	10	2804, 2393, 1874, 1635, 1322, 1128, 937, 844, 738, 670
30	P_{41}	5	1483, 1274, 962, 718, 571
31	P_{42}	6	1837, 1433, 991, 867, 717, 567
32	P_{43}	5	1229, 926, 812, 717, 636
33	P_{45}	5	1378, 1173, 844, 738, 512
34	P_{46}	4	1229, 867, 717, 567
35	P_{47}	4	2638, 2368, 2139, 1775
36	P_{48a}	5	1229, 926, 812, 675, 567
37	P_{44},P_{49},P_{50}, P_{51b}, P_{52}	5	1229, 991, 867, 763, 636
38	P_{53}	4	2368, 2038, 1775, 1443

Table 3. RAPD types of the SNP isolates

SNo	Codes of theisolates	No. of RAPDb ands produced	Number of the base pairs (bp)
1	S_1	5	2291, 1811, 1409, 787, 465
2	S_{2a}	5	2079, 1733, 1355, 787, 465
3	S_{2b}	3	1409, 1087, 477
4	S_3	8	1255, 1074, 962, 807, 706, 663, 520, 417
5	S_4	6	1723, 1512, 1283, 1187, 953, 755
6	S_5	8	1792, 1656, 1534, 1235, 1012, 893, 817, 771
7	S_6, S_7	5	1361, 1074, 754, 464, 375
8	S_8	7	1764, 1419, 754, 684, 586, 395, 339
9	S_{10}	10	3793, 2181, 1733, 1466, 1256, 1087, 950, 867, 701, 538,
10	S_{13}	4	1733, 1466, 891, 787
11	S_{14}	5	950, 787, 701, 628, 512
12	S_{15a}	11	1983, 1591, 1355, 1167, 982, 891, 837, 787, 701, 663, 448
13	S_{15b}	5	1304, 1087, 891, 742, 663
14	S_{16}	6	2291, 1894, 1466, 1256, 787, 701
15	S_{20}	4	807, 586, 339, 294
16	S_{21}	4	706, 586, 440, 280
17	S_{23}	4	1594, 1431, 601, 473
18	S_{24}	3	930, 726, 647
19	S_{25}	5	2277, 1170, 819, 755, 522
20	S_{26}	5	2776, 1154, 980, 794, 519
21	S_{27a}	7	1170, 930, 819, 560, 505, 443, 320
22	S_{27b}	5	1784, 1431, 1291, 891, 392
23	S_{28a}	8	2990, 2009, 1784, 1431, 1291, 972, 891, 647
24	S_{28b}	8	1594, 1359, 1229, 1017, 930, 726, 624, 458
25	S_{30}	8	1866, 1235, 1080, 893, 794, 709, 603, 545
26	S_{31}	10	2028, 1593, 1325, 1235, 1080, 980, 841, 709, 653, 519
27	S_{32}	8	1288, 1049, 866, 791, 694, 638, 588, 433
28	S_{33}	5	1288, 1049, 827, 612, 449
29	S_{35b}	7	1103, 908, 791, 665, 638, 565, 433
30	S_{37}	6	908, 791, 638, 565, 466, 433
31	S_{38b}	5	1648, 1449, 1234, 1101, 953
32	S_{39}	5	1222, 999, 757, 543, 403
33	S_{41}	1	1283
34	S_9, S_{18}, S_{22}, S_{34}, S_{35a}, S_{36}, S_{38a}		No RAPD bands produced

Table 4. Fragment length polymorphisms among the total ***B. thuringiensis* isolates**

Range of fragment length (bp)	Total number of bands produced	Range of fragment length (bp)	Total number of bands produced
200-300	2	1700-1800	12
300-400	9	1800-1900	6
400-500	25	1900-2000	10
500-600	23	2000-2100	7
600-700	34	2100-2200	6
700-800	50	2200-2300	4
800-900	46	2300-2400	4
900-1000	21	2400-2500	1
1000-1100	17	2500-2600	4
1100-1200	13	2600-2700	1
1200-1300	32	2700-2800	1
1300-1400	14	2800-2900	1
1400-1500	17	3100-3200	1
1500-1600	20	3300-3400	1
1600-1700	8	3700-3800	1

Calculation of discriminatory index value (D)

Of 86 isolates typed, 72 different polymorphics were found. The discriminatory index value (D) was calculated using the formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

The D value was found to be 0.9901.

Discussion

All the soil samples in this study were collected from high altitude mountain area - Khumbu base camp of Everest region, Nepal expecting mainly cold tolerating strains of *B. thuringiensis.* The genome of each *B. thuringiensis* is unique and is basic to all DNA analysis aimed at identification (Belkum *et al*. 1994). Based on this assumption RAPD-PCR was optimized to study genetic diversity of the *B. thuringiensis* isolates from Khumbu region of Nepal. As RAPD-PCR has higher discrimination power than any other conventional techniques (Lechner *et al*.1998, Daffonchio *et al*. 1999, Robert & Crawford 2000, Brousseau *et al*. 1993, Puenti-Redondo *et al*. 1999) and opens a new horizon with reproducible data, it is considered as a doorway for any genetic analysis to perform. RAPD assay resulted in a clear separation of the psychrotolerant *B. cereus* strains (Lechner *et al*. 1998).

A series of 100 decamer primers from codes 201 to 300 (obtained from UBC) were tested for RAPD-PCR. It has been shown that the optimal length of primers used in RAPD analysis is approximately eight nucleotides. Primers longer than 10 nucleotides have less discriminating power, which again is strongly dependant on the annealing temperature (Belkum 1994). Nine primers, with GC% of 50-80, were found to amplify the target sequences with reproducible polymorphism to differentiate the *B. thuringiensis* strains. The primers with high GC content (70-80%) were found to produce more polymorphisms compared to those of low GC content (50-60%). PCR products are visualized by ethidium bromide staining (0.2-0.5 ìg/ml of gel) of electrophoretically separated DNA in agarose gel. Fingerprints are recorded as banding patterns and comparisons made by visual inspections using standard scales. Standard molecular markers (λ DNA *Hin*d III digest and ϕ X 174 phage DNA type II digest) were used. Each primer amplified polymorphisms ranged from two to ten over a range of 300 bp to 3 kbps. The bands were found reproducible for different independent DNA preparations from respective *B. thuringiensis* strains.

Each primer yielded RAPD patterns that were unique to strains of the *B. thuringiensis* isolates to be differentiated subsequently. Primer 284 from the series was selected best to type the *B. thuringiensis* isolates for polymorphic study. Of the total amplified products, maximum number of the product size ranged from 300 bp to 2 kb; with highest number of 50 bands within 700 to 800 bp, followed by 46 bands within 800 to 900 bp, 34 bands within 600 to 700 bp and 32 bands within 1200 to 1300 bp. Similar band patterns suggest that the strains are closely related to each other within each group. However, the data must be interpretative with caution since PCR bands of similar size do not necessarily mean that the molecules are identical in sequence (Brousseau *et al*.1993). As the strains were isolated from high altitude mountain region of the country, the maximum amplified genomes may represent cold tolerant genes common to all or the crystal endotoxin producing genes. However, for the best knowledge, such study for cold tolerance was not found yet and the data were not compared to any reference but predicted to contain common or consensus bands (or may be sequences) for cold tolerance so as to adopt the organisms in a given ecological niche.

Vogel *et al*. (1999) tested the usefulness of genomic typing methods *viz*. RAPD analysis and ribotyping with conventional serotyping for three collections of well defined clinical *E. coli* isolates and found that RAPD had the highest discriminatory capacity. In order to determine the index of discrimination (Hunter 1990, Hunter & Gaston 1998), all the 86(*N*) crystal producing *B. thuringiensis* strains were classified into 71(*s*) types with two sets of five similar band patterns (n_{14} and n_{37}), one set of two similar band pattern (n_{45}) and 68 sets of a single band pattern (n_1-n_{13}, n_{15}-n_{36}, n_{38}-n_{44}, and n_{46}-n_{71}). Altogether six *B. thuringiensis* isolates were not found to contain amplifying region in their genome using 284 primers and were classified in the 72nd type as n_{72}.

The RAPD-PCR, used to categorize the *B. thuringiensis* strains isolated from Khumbu region of Nepal, was found to discriminate the organisms with 99.01% confidence (D = 0.9901). The level of confidence satisfied the conclusion made by Vogel *et al*. (1999) to define RAPD analysis had the highest discriminatory capacity for typing *E. coli* isolates. Similarly, while working for RAPD-typing of *Pseudomonas aeruginosa* from cystic fibrosis

patients, Mahenthiralingam *et al*. (1996) stated that, in general, despite alteration in the expression of mucoid exopolysaccharide, bacterial motility, and acquisition of a serum-sensitive phenotype, the RAPD fingerprints of sequential isolates remain stable, suggesting that these changes result from phenotypic adaptation of the primary colonizing isolates. With these findings, the polymorphisms set by RAPD to quantify the diversity of *B. thuringiensis* strain isolates from Khumbu region of Nepal can be confidently defined as rich one.

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References

Belkum, A. 1994. DNA fingerprinting of medically important microorganisms by use of PCR. *Clinical Microbiology Reviews* **7**: 174-184.

Ben-Dov, E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, R. Manasherob, A. Khamraev, E. Troitskaya, A. Dubitsky, N. Berezina and Y. Margalith. 1997. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl. Environ. Microbiol*. **63**: 4883-4890.

Brousseau, R., A. Saint-Onge, G. Prefontaine, L. Masson and J. Cabana. 1993. Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Applied and Environmental Microbiology*. **59**: 114-119.

Claus, D. and R.W.C. Berkeley. 1986. Genus *Bacillus* Cohn 1872. In *Bergey's*. *Manual of Systematic Bacteriology* Vol. 2. (ed. Sneath, P.H.A.) Baltimore, M.D: Williams & Wilkins. pp. 1105-1138

Daffonchio, D., S. Borin, G. Frova, R. Gallo, E. Mori, R. Fani and C. Sorlini. 1999. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. *Appl. Environ. Microbiol*. **65**: 1298-1303.

Dulmage, H.T. 1970. Production of spore-delta-endotoxin complex by variants of *Bacillus thuringiensis* in two fermentation media. *Journal of Invertebrate Patholog* **16**: 385-389.

Hunter, P.R. 1990. Reproducibility and indices of discriminatory power of microbial typing methods. *Journal of Clinical Microbiolog* **28**: 1903-1905.

Hunter, P.R. and M.A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of simpson's index of diversity. *Journal of Clinical Microbiolog* **26**: 2465-2466.

Lechner, S., R. Mayr, K.P. Francis, B.M. Pruss, T. Kaplan, E. Wiessner-Gunkel, G.S. Stewart and S. Scherer. 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *International Journal of Systematic Bacteriolog* ***48*** (4): 1373-1382.

Mahenthiralingam, E., M.E. Campell, J. Foster, J.S. Lam and D.P. Speert. 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology*. **34**: 1129-1135.

Martin, P.A. and R.S. Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol* **55**: 2437-2442.

Mehling, A., U.F. Wehmeier and W. Pieperberg. 1995. Application of random amplified polymorphic DNA (RAPD) assays in identifying conservercer regions of actinomycete genomes. *FEMS microbiol. Lett.* **128**: 119-126.

Puente-Redondo, V.A., N.G. Blanco, C.B. Gutierrez-Martin, F.J. Garcia-Pena and E.F.R. Ferri 2000. Comparision of different PCR approaches for typing of *Francisella tularensis* strains. *Journal of Clinical Microbiology* **38**: 1016-1022

Roberts, M.A. and D.L. Crawford. 2000. Use of randomly amplified polymorphic DNA as a means of developing genus- and strain-specific *Streptomyces* DNA probes. *Applied and Environmental Microbiology* **66**:2555-2564.

Sambrrok, J., E.F. Fritsch, T. Maniatis. 1989. Molecular cloning: A laboratory manual. (2nd Ed). cold spring harbor laboratory press, *Cold Spring Harbor*, New York

Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler and D.H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biololgy Reviews* **62**: 775-806.

Shrestha, U.T., G.S. Sahukhal, S. Pokharel, K.B. Tiwari, A. Singh and V.P. Agrawal. 2006. Delta endotoxin immunocrossreactivity of *Bacillus thuringiensis* isolates collected from Khumbu base camp of Mount Everest region. *Journal of Food Science Technology Nepal* **2**: 128-131.

Vogel, L, E. HMM, M. Mindehoud and L. Dijkshoom. 1999. Epidemiologic typing of *Escherichia coli* using RAPD analysis, ribotyping and serotyping. *Journal of Clinical Microbiology* **6**: 82-87.

Yoon, J.M. and G.W. Kim. 2001. Randomly amplified polymorphic DNA-polymerase chain reaction analysis of two different populations of cultured Korean catfish *Silurus asotus*. *Indian Academy of Sciences* **26**: 641–647.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**: 6531-6535.