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Isolation and characterization of *Bacillus thuringiensis* Berliner from soil, leaf, seed dust and insect cadaver

R. ASOKAN[*#] and **PUTTASWAMY**
Department of Entomology, University of Agricultural Sciences
GKVK, Bangalore 560 065, Karnataka, India
E-mail: asokan@iihr.ernet.in

ABSTRACT: **Improved methods of isolation of *Bacillus thuringiensis* (Bt) has resulted in the isolation of novel strains with new host range, increased toxicity over the existing isolates and as source of isolation of novel genes. The potent and novel genes of Bt such as vegetative insecticidal proteins (Vip) and binary toxins are currently used for production of insect resistant transgenic plants. In the present investigation Bt has been isolated from the following sources *viz*., soil (19 out of 38 locations), leaf (one out of 12 samples), seed dust (two out of seven samples) and insect cadavers (two out of nine insect species). Average population of the Bt isolates varied from 0.24 x 10^5 cfu/g in soil; 0.04 cfu/cm^2 in leaf; 0.09 x 10^2 cfu/g in seed dust and 1.38 x 10^5 cfu/larva in insect cadaver. As far as the number of Bt isolates obtained from different sources 28, one, two and two isolates were obtained from soil, leaf, seed dust & insect cadaver, respectively. Out of the total 33 isolates 18, 10 and five isolates produced bipyramidal, rhomboidal and spherical crystals, respectively. Preliminary toxicity studies showed that the 18 isolates that produced bipyramidal crystals were toxic to the five-day-old larvae of *Plutella xylostella* (Linnaeus). PCR screening of the above isolates using hemolysin specific primers showed that the objectionable exotoxin, hemolysin was present in only one isolate, DVu-1 obtained from the seed dust of *Vigna unguiculata* (Linnaeus).**

KEY WORDS: *Bacillus thuringiensis*, hemolysin, isolation, preliminary toxicity, types of crystals

INTRODUCTION

The various crystal protein toxins (Cry) produced by Bt have been successfully used in pest management programmes both in agriculture and public health with no effect on non-target organisms. In the recent years, there is a renewed interest in isolating potent Bt strains with increased host spectrum and also a source of isolation of novel genes. In this regard, occurrence of Bt from different sources such soil, leaf, seed dust has been reported to be less. The sodium acetate based selection medium developed by Travers *et al.* (1987) had tremendously improved isolation of Bt from soil and also from other sources. Presently, Bt has been isolated from different sources, *viz*. fresh water

* Corresponding author
# Presently at the Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta Lake (PO), Bangalore 560 089

(Ichimatsu *et al.*, 1998), saw dust (Bravo *et al.*, 1980), cured tobacco leaves (Kaelin and Gadani, 2000), rice bran (Jung *et al.*, 1998), stored products, compost, phylloplane (Bernhard *et al.*, 1997), marine sediments (Maeda *et al.*, 2000) and in ancient glacial ice (Christner *et al.*, 2003). Host range of Bt includes Lepidoptera, Coleopteran, Diptera, Acarina, Protozoa, Hymenoptera, Trematode and Nematodes (Crickmore *et al.*, 1998). Intensive screening programmes have yielded many new genes such as vegetative insecticidal proteins (Vip) (Estruch *et al.*, 1996). Therefore, there is a need for continued effort to isolate more potent Bt strains from diverse sources. In the present study, we report isolation of Bt from different sources such as soil, leaf, seed dust and insect cadaver; types of crystal produced; toxicity to the five-day-old larvae of *P. xylostella* and PCR screening of the Bt isolates for presence of the exotoxin, hemolysin.

MATERIALS AND METHODS

I. Collection of samples

Soil

One gram of soil was collected at depth of five cm from uncultivated land, across 38 locations in different agro climatic zones of Karnataka and stored in sterile polythene bags (10 x 5 cm) until further use (Table 1).

Table 1. Locations in Karnataka selected for soil sampling *Bt* isolates

Sl no.	Agro climatic zones	Place of sample collection
1	Zone 1 (North Eastern Transition)	Bidar (BDR)*
2	Zone 2 (North Eastern Dry)	Gulbarga (GUL), Raichur (RCR)
3	Zone 3 (Northern Dry)	Bijapur (BJR), Bellary (BRY)
4	Zone 4 (Central Dry)	Chitradurga (CGA), Davanagere (DGE)
5	Zone 5 (Eastern Dry)	Tumkur (TMR), Bangalore (BLR)
		Hessaraghtta (BLRA),Thirumalapura (BLRB)
		Hurulichikanahalli (BLRC), Shivakote (BLRD)
		Bylekere (BLRE), Lakshmipura (BLRF)
		Madhukur (BLRG), Chikasandra (BLRH)
		Kesthur (BLRI), Kolar (KLR)
6	Zone 6 (Southern Dry)	Mandya (MDA), VC Farm (MDAA)
		Holalu (MDAB), Mysore (MYR)
7	Zone 7 (Southern Transition)	Hassan (HSN), Shimoga (SMA)
8	Zone 8 (Northern Transition)	Belgaum (BLM), Dharwad (DWD)
9	Zone 9 (Hilly)	Gonikoppa (CRGA), Chethalli (CRGB)
		Madikeri (CRGC), Veerajpet (CRGD)
		Chikamagalur (CMLR)
10	Zone 10 (Coastal)	Mangalore (MLR), Puttur (MLRP)
		Sulliya (MLRS), Karwar (KWR)
		Kumata (KMTA), Ankola (ALA)

* Letters in the parentheses represent the abbreviation used for the place of soil collection.

Leaf

Three leaves each from some important horticultural crops, *viz. Mangifera indica, Psidium gujava, Ziziphus* sp., *Annona squamosa, Punica granatum, Citrus aurantifolia, Manilkara zapota, Vitis vinifera, Brassica oleraceae, Lycopersicon esculentum, Solanum melongena* and *Abelmoscus esculentus* were collected and stored in sterile polythene bags (10 x 5 cm) until further use.

Seed dust

One gram of grain dust form *Vigna unguiculata, Abelmoscus esculentus, Lagenaria* sp., *Luffa acutangula, Dolichos lab lab, Raphanus sativus and Amaranthus* sp. were collected from the seed processing unit (IIHR, Bangalore) and stored in sterile polythene bags (10 x 5 cm) until further use.

Insect cadaver

Regular survey was conducted in the horticultural farm (IIHR, Bangalore) and neighbouring villages. Dead/diseased/ moribund larvae of *Plutella xylostella, Acherontia styx, Leucinodes orbonalis, Papilio demoleus, Crocidolomia binotalis, Helicoverpa armigera, Achaea janata, Spodoptera litura and Myllocerus subfaciatus* were collected in sterile glass vials (50 x 10 cm) and stored at 4°C until further use.

II. Isolation of Bt

Soil and Seed dust

Isolation of Bt was carried out according to Travers *et al.* (1987). One gram of the soil was mixed with 10 ml of Luria broth buffered with 0.25 M sodium acetate, rotated at 250 rpm for four hours at 30°C and heat shocked at 80°C for three minutes. Serial dilutions (10^{-1} to 10^{-4}) were made in sterile distilled water and 100 μl of each dilution was spread on Luria agar and incubated at 30°C overnight. There were three replications maintained for each dilution. Chalky white colonies were picked up and plated on T3 medium and incubated at 30°C for 72 hours. All the selected colonies were further purified employing single colony isolation and maintained on nutrient agar at 4°C until further use.

Leaves

The leaves were washed in sterile distilled water to remove the superficially adhering microflora and taken in 100 ml sterile double distilled water and rotated at 250 rpm, 30°C for four hours. The resulting suspension was centrifuged at 10000 rpm, at 4°C for 15 minutes and the supernatant was discarded. Five ml of Luria broth buffered with 0.25 M sodium acetate was added to the pellet and the rest of the procedure of isolation was same as that of soil.

Insect cadaver

The larvae were surface sterilized using rectified spirit and individual larvae were homogenized in a microfuge in one ml of Luria broth buffered with 0.25 M sodium acetate. The other isolation procedure was the same as above.

Identification of crystalliferous Bt isolates and types of crystals produced

Spore-crystal mixture of the above selected colonies were stained with crystal violet and observed under oil immersion (100x) for the production of crystals. The crystalliferous isolates were further screened in a phase contrast microscope to determine the type of crystals produced by the crystalliferous isolates.

III. Determination of Bt isolation index

Population of the total *Bacillus* isolates and crystalliferous Bt isolates were determined for each sample by taking into account of the initial quantity of the sample taken, dilution, surface area (in the case of leaf). The population was expressed as number of colony forming units (cfu)/ unit area (per gram for soil and seed dust; cm^2 for leaf and per larva for insect cadaver). Bt isolation index was worked out by dividing the population of crystalliferous Bt isolates by the total population of *Bacillus* for each sample collected from different sources.

IV. Preliminary toxicity studies on the crystalliferous isolates to *P. xylostella*

Larvae of *P. xylostella* were collected from cabbage crop with no previous application of any *Bt* formulations an the stock culture was maintained at the Department of Entomology, University of Agricultural Sciences, GKVK, Bangalore on potted cabbage plants in wooden cage (45 x 45 x 45 cm). Culturing was carried out at temperature ranging from 25 to 32°C and relative humidity of 72 to 90 per cent and five-day-old larvae were used for bioassay studies.

Preliminary toxicity studies were conducted using the 100 µl of spore-crystal mixture, which was applied uniformly on both sides of the cabbage leaves. Ten-number of five-day old larvae of *P. xylostella* was released in each Petri plates. There were three replications for each treatment and an untreated control was maintained. Mortality of the treated larvae was recorded at 24 hours interval for 72 hours and the per cent mortality was subjected to ANOVA after *arcsine* transformation.

VI. Screening for objectionable exotoxin, hemolysin in the crystalliferous Bt isolates

PCR screening of the above 33 isolates were carried out to determine for the presence of hemoysin employing the primers, *viz. hbl*A1 - GCT AAT GTA GTT TCA CCT GTA GCA AC, *hbl*A2 – AAT GAT GCC ACT GCG TGG ACA TAT AA with an expected product size of 883 bp (Ryan *et al.*, 1997). The reaction mix contained the following components, *viz.* template – 5 µl, 10x buffer – 2.5 µl, dNTP mix – 0.5 µl, *Taq* polymerase – 0.5 µl, primers (*hbl*A1 & *hbl*A2) – 1.0 µl each, sterile distilled water – 14.5 µl. PCR was carried out in a 96 well Thermal cycler with the following reaction conditions; first five cycles of initial denaturation – 94°C for 4 min, denaturation – 94°C for 30 sec, annealing – 70°C for 1 min, extension – 72°C for 1.5 min; and 35 cycles of denaturation – 94°C for 30 sec, annealing – 65°C for 1 min, extension – 72°C for 1.5 min and final extension – 72°C for 5 min. The PCR products were resolved in an agarose gel (1%) in TBE (1x), 150 volt hours and stained with ethidium bromide (1 µg/ml) and visualized in a transilluminator.

RESULSTS AND DISCUSSION

I. Isolation of crystalliferous Bt isolates and types of crystals produced

Microscopic observations of the lysed culture of different isolates revealed that a total of 33 crystalliferous Bt isolates were obtained from different sources of sample collection, *viz.* soil, leaf, seed dust and insect cadaver. With respect to the number of Bt isolates obtained from different sources a highest number of 28 isolates were obtained from soil. A maximum number of two isolates were obtained from Raichur, Bellary, Tumkur, Kolar, Mysore, Hassan, Dharwad, Chikamagalur and Karwar. A maximum number of single isolate was obtained from Bidar, Davanagere, Bangalore, Bylekere, Lakshmipura, Mandya, Belgaum, Gonikoppa, Madikeri and Mangalore (Table 2). Only single isolate was obtained from the leaves of *A. squamosa*. In the case of seed dust, two isolates, one each from *V. unguiculata* and *L. acutanguala* was isolated. Two isolates one each from *P. xylostella* and *Papilio demoleus* was obtained (Table 2). Kaelin and Gadani (2000) reported that Bt could be isolated form 10 out of 132 samples implying Bt was not present uniformly. This could be the why crystalliferous Bt was isolated only from 19 out of 38 soil samples. This could be applicable for other sources such as leaf, where Bt was isolated from only one plant species, *A. squamosa* out of 12 species studied. In the case of seed dust, Bt was not present in five seed dust samples. In the case of insect cadaver Bt was not recorded in seven insect species.

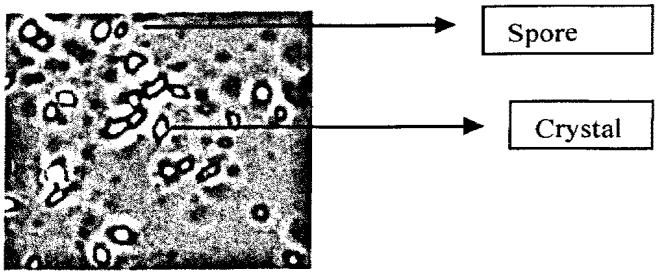
As far as the types of crystals produced by above 33 isolates, three crystal types, *viz.* bipyramidal, rhomboidal and spherical were evident and bipyramidal crystal was most predominant in 18 isolates (Fig.1). The next predominant crystal type was rhomboidal observed in 10 isolates followed by spherical type in only in five isolates (Table 2). Kaelin (2000) reported that out of 24 isolates studied 15 isolates produced bipyramidal crystals as compared to other crystal types. When the crystal types were regrouped according to the sources from which Bt isolates were obtained, 14,

Table 2. Preliminary toxicity of *B. thuringiensis* isolates obtained from different sources

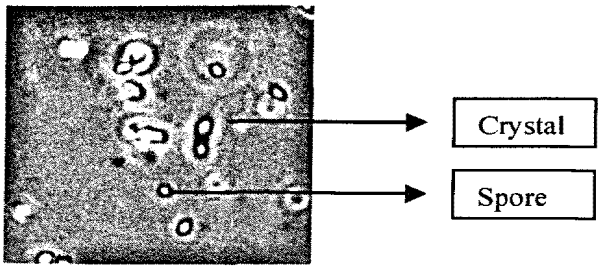
Sl no.	Source of sample collection	Bt isolation index	Isolate No.	Larval mortality(%)	Type of insecticidal crystal produced
1	**Soil**Bidar	0.25	BDR-3	100.0 (90.0)[b]	Bipyramidal
2	Raichur	0.26	RCR-1	100.0 (90.0)[b]	Bipyramidal
			RCR-6	100.0 (90.0)[b]	Bipyramidal
3	Bellary	0.26	BRY-3	Non toxic	Rhomboidal
			BRY-4	63.3 (61.1)[a]	Bipyramidal
4	Davanagere	0.33	DGE-2	Non toxic	Rhomboidal
5	Tumkur	0.15	TMR -5	Non toxic	Spherical
			TMR-10	30.0 (22.4)[a]	Bipyramidal
6	Bangalore	0.10	BLR-4	Non toxic	Rhomboidal
7	Bylekere	0.10	BLRE-1	Non toxic	Rhomboidal
8	Lakshmipura	0.33	BLRF-2	36.6 (33.2)[a]	Bipyramidal
9	Kolar	0.13	KLR-2	Non toxic	Rhomboidal
			KLR-15	Non toxic	Rhomboidal
10	Mandya	0.09	MDA-5	46.6 (44.9)[a]	Bipyramidal
11	Mysore	0.20	MYR-5	50.0 (38.6)[a]	Bipyramidal
			MYR-9	Non toxic	Rhomboidal
12	Hassan	0.28	HSN-3	53.3 (48.3)[a]	Bipyramidal
			HSN-4	Non toxic	Spherical
13	Belgaum	0.33	BLM-3	Non toxic	Spherical
14	Dharwad	0.17	DWD-1	40.0 33.2)[a]	Bipyramidal
			DWD-5	Non toxic	Rhomboidal
15	Gonikoppa	0.06	CRGA-6	Non toxic	Rhomboidal
16	Madikeri	0.25	CRGC-2	100.0 (90.0)[b]	Bipyramidal
17	Chickamagalur	0.19	CMLR-8	43.3 (41.9)[a]	Bipyramidal
			CMLR-9	46.6 (28.2)[a]	Bipyramidal
18	Mangalore	0.25	MLR-4	Non toxic	Spherical
19	Karwar	0.20	KWR-2	100.0 (90.0)[b]	Bipyramidal
			KWR-3	Non toxic	Spherical
20	**Leaf***A. squamosa*	0.18	IAs – 1	Non toxic	Rhomboidal
21	**Seed dust***V. unguiculata*	0.10	DVu – 1	53.3 (47.9)[a]	Bipyramidal
22	*L. acutangula*	0.20	DLa – 1	46.6 (41.7)[a]	Bipyramidal
23	**Insect cadaver***P. xylostella*	-	KPx – 1	100.0 (90.0)[b]	Bipyramidal
24	*P. demoleus*	-	IPd – 1	100.0 (90.0)[b]	Bipyramidal
25	*Bt kurstaki*	-	HD-1	100.0 (90.0)[b]	Bipyramidal

CD (p=0.05) = 40.58

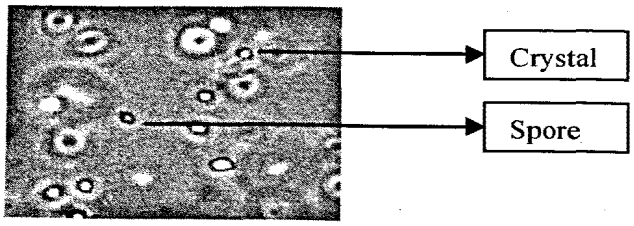
nine and five isolates produced bipyramidal, rhomboidal and spherical types, respectively, from soil. Only bipyramidal crystals were observed in the four isolates that were obtained from seed dust and insect cadaver. In the case of leaf, rhomboidal type crystal was observed (Table 2). According to Zelanzny *et al.* (1994) the shape of the crystal depended on the protoxin composition i.e. bipyramidal crystals contain Cry I type of polypeptides, rhomboidal crystals contain Cry II and spherical crystals contain Cry 4 type of protoxins that are toxic to different orders of insects.



Bipyramidal



Rhomboidal



Spherical

Figure1. Phase contrast images of insecticidal crystals observed in different Bt isolates

II. Bt isolation index

The average population of total *Bacillus* was 2.54 x 103 cfu/g, 0.12 cfu/cm^2 and 1.29 x 10^2 cfu/g in soil, leaf and seed dust, respectively. There was no acrystalliferous *Bacillus* was observed in the insect cadaver. Soil had the highest background population of *Bacillus* as compared to the other sources. As far as the population of crystalliferous Bt was concerned a highest population was obtained from the insect cadaver (1.38 x 10^5 cfu/ larva), while leaf was the poor source for isolation of Bt (0.04 cfu/ cm^2). An average population of 0.24 x 10^3 cfu/g and 0.09 x10^2 cfu/g was recorded from soil and seed dust, respectively.

Determination Bt isolation index for soil samples collected from different locations showed that highest of 0.33 was recorded for three locations, *viz.* Davanagere, Lakshmipura and Belgaum and a lowest value of 0.06 was obtained for Gonikoppa. In the case of leaf, *A. squamosa* recorded an isolation index of 0.18, while in the seed dust Bt isolation index ranged from 0.10 in *L. acutanguda* and 0.20 in *V. unguiculata* (Table 2). Travers *et al.* (1987) showed that sodium acetate selection method reduced the background population from 6.34 x 10^6 to 8.0 x 10^3 cfu/g of soil. But the only lacuna of this method was the possible elimination of some stains of Bt that were not amenable for sodium acetate selection. In addition to the above Bt isolation index for different samples collected from different sources primarily dependent on the background *Bacillus* population and also possibly influenced by other parameters like pH and organic matter in the soil. In the present study occurrence of Bt could not be isolated from all the samples collected from different sources and therefore occurrence of Bt is random and many earlier workers (Bernhard *et al.*, 1997; Hongyu *et al.*, 2000a) showed that soil was the major source for the isolation of Bt, which is also observed in the present investigation also.

III. Preliminary toxicity studies

This study showed that 18 isolates were toxic to the five-day-old larvae of *P. xylostella* out of the 33 isolates tested. Correlating the type of crystals produced and the toxicity, it was observed that isolates that produced bipyramidal crystals only were toxic to *P. xylostella*. Among the 18 isolates,

three isolates, *viz.* BDR-3, RCR-1, RCR-6, CRGC-2, KWR-2 (isolated from soil) KPx-1 and IPd-1 (isolated from insect cadaver) caused 100 per cent mortality, while the mortality caused by other 11 isolates were statistically non significant (Table 2). There was no mortality in the untreated control. Detailed bioassay studies will reveal the LC_{50} for each of the isolate which could be compared to the International standard for Bt subspecies *kurstaki*, HD-1-S-1980, for computing relative toxicity. Travers *et al.* (1987) showed that Bt isolates that produced bipyramidal crystals were lepidopteran active as compared to the Bt isolates that produced other types of crystals, viz. spherical, oval, rhomboidal and irregular. Therefore Bt isolates that produced rhomboidal (10 isolates) and spherical (five isolates) could be active on other orders such as coleopteran, diptera etc. It is interesting to observe that not all the isolates that were obtained from the same location were toxic to *P. xylostella*, except for RCR-1 and RCR-6 that were isolated from Raichur, which caused 100 per cent mortality. In the case of other locations, either there was a variation in toxicity to *P. xylostella* or both toxic and non-toxic isolates to *P. xylostella* was obtained. This implied different strain of Bt isolates could be obtained from a given location.

IV. Screening for the presence of hemolysin

PCR screening of the 33 isolates that were isolated in this study showed that hemolysin was found in only one isolate, DVu-1, which was isolated from the seed dust of *V. unguiculata* (Figure 2). It is important to screen the potential Bt isolates before making a suitable formulation since hemolysin has broad-spectrum hemolytic activity in the non-target organisms including humans. Jackson *et al.* (1995) showed that Bt was isolated in a gastroenteritis outbreak, which produced hemolysin. Similarly Damgaard *et al.* (1998) isolated enterotoxin-producing strains of Bt from various food sources. Therefore even if a particular Bt isolate is highly toxic it should devoid of production of the above toxin. In this context 32 out of the 33 isolates are safer and some of the potential isolates like BDR-3, RCR-1, RCR-6, CRGC-2, KWR-2, KPx-1 and IPd-1 could be scaled up as spray formulation or as a source of isolation of potent crystal protein genes.

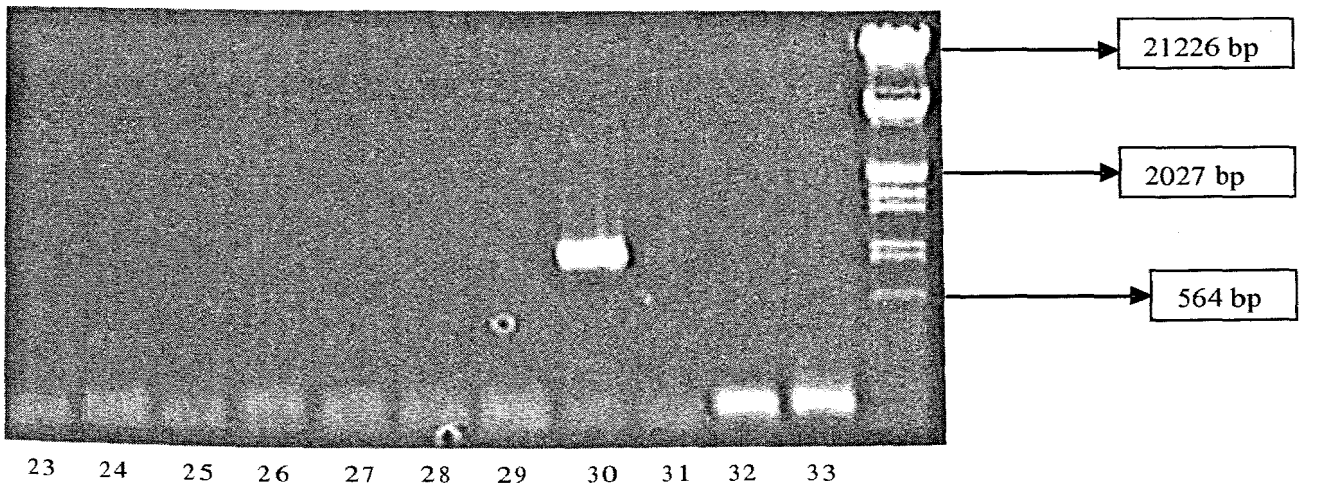


Figure 2. PCR screening for the presence of hemolysin in different isolates of Bt
(Lanes: 23-CRGC-2; 24-CMLR-8; 25-CMLR-9; 26-MLR-4; 27-KWR-2; 28-KWR-3; 29-IAs – 1; 30-DVu – 1; 31-DLa – 1; 32-KPx – 1; 33-IPd – 1)

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