

## A comparative study on biodegradation of chlorpyrifos by wild *E. coli* and *Pseudomonas fluorescens* bacterial isolates inhabiting different ecosystems of Kashmir valley

Imtiyaz Murtaza<sup>1,\*</sup>, Bushra<sup>1</sup>, Sageera Showkat<sup>1</sup>, Shah Ubaid-Ullah<sup>2</sup>, Omi Laila<sup>1</sup>, Sumyra Majid<sup>1</sup>, Neyiaz A. Dar<sup>1</sup>, Mukhtar Ahmad<sup>3</sup> and Girish Sharma<sup>4</sup>

<sup>1</sup>Biochemistry and Molecular Biotechnology Laboratory, Biochemistry Section, Division of Basic Sciences, SKUAST-K, Shalimar Campus, Srinagar 190 025, India

<sup>2</sup>Department of Biotechnology, School of Life Sciences, Central University of Kashmir, Srinagar 190 004, India

<sup>3</sup>RCRQ Laboratory, SKUAST-K, Shalimar Campus, Srinagar 190 025, India

<sup>4</sup>Department of Biotechnology, Amity University, Sector-125, Noida 201 313, India

**Among 1081 naturally occurring wild isolates examined for *E. coli* and *Pseudomonas fluorescens*, EC1 (*E. coli*) from Dal Lake (Srinagar district) and PF1 (*P. fluorescens*) from soil samples of Ganderbal district showed maximum tolerance (11 mg/ml) towards chlorpyrifos. The high performance liquid chromatography (HPLC) based chlorpyrifos (CP) degradation analysis demonstrated that each isolate degraded chlorpyrifos much more efficiently than the reference strain *E. coli* MTCC-533 used in the current study. The present study suggests that EC1 and PF1 bacterial isolates inhabiting different ecosystems, degrade chlorpyrifos efficiently via genetic determinants and OPP enzymatic system and provide strong basis for development of bioremediation strategies in the area.**

**Keywords:** Bioremediation, chlorpyrifos, *E. coli*, HPLC, *Pseudomonas fluorescens*, resistance.

INSECTICIDES play an important role in increasing the agricultural yield. While less than 1% of the pesticides are enough to kill the pests, the remaining pesticides especially organophosphates enter the soil, ground and surface water and continuously pollute such ecosystems<sup>1</sup>. Therefore, there is an immediate need to remediate such polluted environments by aggressively adopting bioremediation processes<sup>2</sup>. India loses about 30% of its agriculture produce due to pest infestations, and to ensure food-for-all, indispensable use of pesticides has become common practice<sup>3</sup>. Continuous use of such pesticides has raised some serious concerns as their persistence and solubility in a particular habitat causes serious environmental pollution problems<sup>4</sup>. Among the various pesticides currently used, chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-

trichloro-2-pyridinyl)] (CP) represents one of the most commonly used agricultural organophosphorus (OP) insecticides, which controls a broad spectrum of insects<sup>5</sup>. Owing to its widespread use in agriculture, there is every chance of ingesting CP-contaminated foods; chlorpyrifos residues are being detected in fruits, vegetables, cattle meat, etc.<sup>6,7</sup>.

Chlorpyrifos pesticides easily enter the human food chain and affect more people than carcinogenic air pollutants such as polycyclic aromatic hydrocarbons (PAHS)<sup>8,9</sup>. The use of chlorpyrifos has been vastly restricted in the US and some European countries; however, it is still widely used in developing countries like India<sup>10</sup>. An increasing trend in the incidence of primary malignant brain tumours and familial gliomas in orchard farmers, pesticide handlers, orchard-farm workers and orchard-residents due to exposure to toxic pesticides especially chlorpyrifos<sup>11</sup> was reported from the Kashmir valley.

To reduce the negative impacts of such toxic pollutants in the environment, it is desirable to degrade or at least quickly deactivate them in the environment especially by using naturally occurring bacterial microorganisms<sup>12,13</sup>. Such types of processes may contribute to the maintenance of a safe environment. CP degrading microorganisms use chlorpyrifos as a source of phosphorus and carbon for their growth, and this phenomenon is achieved by using hydrolytic and oxidative enzymes present in them<sup>5</sup>. One of the key enzymes, i.e. organophosphorous phosphatase (OPP), involved in chlorpyrifos degradation has been reported to be present both in the intracellular and the extracellular fractions of different bacterial species<sup>14,15</sup>. In order to exploit the naturally occurring pesticide-degrading microbial systems for removal of pollutants from the contaminated systems, it is necessary to understand the ecological, physiological and biochemical requirements of degrading organisms.

Therefore, the main focus of the present study was to isolate chlorpyrifos-resistant wild *Escherichia coli* and *Pseudomonas fluorescens* isolates from crop fields and water bodies of Kashmir valley, characterize the selected chlorpyrifos-degrading isolates, determine the location of chlorpyrifos degrading genetic determinant in the highly resistant isolates, study their biochemical mode of degradation and evaluate their CP degradation patterns by HPLC.

The soil and water samples were collected from different geographical locations of Kashmir Valley having 5–10 years history of chlorpyrifos insecticidal spray in the areas. Soil samples were collected from different fruit and vegetable orchards of Ganderbal and Anantnag districts of Kashmir Valley. Water samples were collected from the world famous Dal lake and Anchar lake as both these water bodies are reported to be polluted with different toxic agents including pesticides. The collected samples were initially screened for the presence of CP-resistant bacterial isolates using MaCconkey, King's

\*For correspondence. (e-mail: imz\_murtaza@hotmail.com)

medium B base (HiMedia, India), and EMB agar (Hi-Media, India) plates amended with 1 mg/ml chlorpyrifos stress. The isolates were further identified by biochemical tests. The control strain used in all the sets of experiments was *E. coli* MTCC-533 from Microbial Type Culture Collection Centre, Chandigarh (India). The chlorpyrifos used was of technical grade.

The maximum concentration of chlorpyrifos tolerated by bacteria isolates was determined by checking the growth on the LB agar plates supplemented with varying concentrations of chlorpyrifos. One hundred  $\mu$ l of overnight grown cultures (*E. coli* and *P. fluorescens*) was spread on solid LB agar plates amended with varying (1–20 mg/ml) concentrations of chlorpyrifos. The plates were incubated at respective optimum temperatures for 24–72 h and any visible growth was checked for determining the minimal inhibitory concentration (MIC). The isolates tolerating the highest concentration of CP were selected for further investigation.

To locate the position of the chlorpyrifos resistance determinant, plasmid curing and plasmid transformation were studied. The wild isolated plasmid DNA from EC1 and PF1 was transferred to *E. coli* DH5 $\alpha$  strain by transformation (CaCl<sub>2</sub>-heat shock method) and the respective pattern of transformants was determined on CP amended Agar plates.

To evaluate the biochemical mode of CP degradation, crude OPP enzyme extracts were made from EC1 and PF1 bacterial isolates as well as from MTCC-533 strain subjected to CP stress. Cells were grown in minimal salt broth containing 0.8 mg/ml chlorpyrifos stress. They were harvested and pelleted after 14 h of overnight growth by centrifugation at 8,000 rpm for 10 min. The supernatant obtained was used to determine extracellular OPP activity. The cell pellet was re-suspended in 50 mM/l Tris-HCl (pH 8) buffer containing 0.1 mM/l phenylmethylsulfonyl fluoride (PMSF) and sonicated using a digital sonifier (Restek, USA) for 10 sec duration with 15 sec incubation on ice. This process of sonication and incubation was repeated several times to ensure complete extraction. The lysate was centrifuged at 10,000 rpm for 30 min and the supernatant collected and used to determine intracellular OPP activity<sup>16</sup>. OPP activity was measured by adding 100  $\mu$ l of crude enzyme to 900  $\mu$ l of Tris-HCl (pH 9) containing 10 mg/ml *p*-nitrophenol phosphate and the mixture was incubated for 10 min at 37°C. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid and 1 ml of 10% Na<sub>2</sub>CO<sub>3</sub>. The liberated yellow coloured end product (*p*-nitrophenol) was measured at 410 nm using spectrophotometer. The OPP enzyme activity assay was carried out in triplicate.

The wild *E. coli* (EC1) and *P. fluorescens* (PF1) isolates tolerating the highest concentration of chlorpyrifos (11 mg/ml) were subjected to HPLC analysis in M9 minimal media containing chlorpyrifos stress. The overnight

grown bacterial cultures (0.1%) were inoculated in different flasks carrying 2 or 5 mg/ml chlorpyrifos stress levels. Simultaneously M9 media with CP stress and devoid of bacterial isolates were used as a negative control and media with MTCC-533 as positive control. The flasks were kept on orbital shaker and cultures allowed to grow for 7 days at 28–37°C. On each alternate day proper aliquots from each flask were processed for HPLC analysis. The collected aliquots were centrifuged on Q-sep 3000 (Restek USA) centrifuge at 7200 rpm for 10 min and supernatant collected. The supernatant and standard reference chlorpyrifos samples were mixed separately with an equal volume of dichloromethane (DCM) and the organic layer obtained was collected and evaporated at room temperature. The residues obtained were dissolved in acetonitrile using rotatory evaporator, filtered through 0.45  $\mu$ m syringe filter and quantified by HPLC (Dionex ultimate 3000 liquid chromatography including quaternary pumps LPG-3400 SD, auto sampler ACC-3000T, column compartment TCC-3000 SD) equipped with photo diode array (PDA) detector (Dd-3000). The separation was performed on C18 column (Phenomenex kinetex 250  $\times$  4.6 mm, 5  $\mu$ m, 100 A). The mobile phase consisted of acetonitrile and water (9 : 1 v/v) and the flow rate was maintained as 1 ml/min. The sample inject volume was 10  $\mu$ l, the column compartment temperature was maintained at 25°C and finally UV detection was done at 205 nm. The total run time was 10 min. Under these operating conditions, the retention time of standard chlorpyrifos was standardized to be 4.32 min. Data analysis was performed by Chromolean software 6.80 version.

Though different efforts are being made to degrade toxic pesticides in various environments, their persistence still creates a lot of human health problems<sup>15</sup>. Based on a recent report in Kashmir, millions of tonnes of pesticides, insecticides and fungicides including chlorpyrifos are being used by orchard farmers. These are sprayed on plants, fruits and the leaves every year and such heavy use of pesticides causes their widespread presence in all elements of the food chain including local water bodies (like Dal Lake), soil, vegetation and fish<sup>17</sup>. In order to cope with such toxic compounds, bacterial isolates from natural environments have proved to be the best bioremediation agents. Water samples collected from Dal Lake and Anchar Lake for isolation of CP resistant bacteria, showed considerable variation with respect to their respective physicochemical properties. Interestingly, in the water samples collected from different locations of Dal Lake ([supplementary data](#)) showed presence of chlorpyrifos and with average concentrations between 0.0143  $\pm$  0.00209 and 0.0201  $\pm$  0.00060 mg/l. The results clearly indicate the excessive use of this pesticide in the vicinity has contaminated the Dal Lake. It has been reported that chlorpyrifos has high soil absorption coefficient than water solubility, and thus microbial degradation of CP in these environments depends on the selection of

appropriate bacterial species<sup>9</sup>. In addition to water, the soil samples collected for isolation of CP resistant bacteria also showed variability with respect to nature of soil, pH and temperature (data not shown). These results indicated that the bacteria found in such different environments may also vary with respect to their CP resistance pattern. Among the selected sample sites, varied number of CP resistant (CP<sup>r</sup>) *E. coli* and *P. fluorescens* was identified (Supplementary Tables A–D). Among the total 1081 CP<sup>r</sup> bacterial isolates collected from four selected sites, interestingly, the maximum number of CP resistant *E. coli* isolates was found in water samples from Dal Lake and maximum number of CP resistant *P. fluorescens* from soil samples was collected from Ganderbal district. The reason for this reported is due to the fact that there is unprecedented use of CP in these areas. Higher chlorpyrifos pesticide residual levels have been reported in fish as well as in human serum samples of local inhabitants of Dal Lake<sup>18</sup>. The results indicate a high indigenous population of chlorpyrifos-resistant bacteria in both terrestrial and aquatic ecosystems of Kashmir Valley. Several reports suggest that exploiting physiological, biochemical and molecular aspects of pollutant transformation in naturally occurring microorganisms will lead to better bioremediation process<sup>19,20</sup>. Among the different *E. coli* and *P. fluorescens* isolates, collected from four different ecosystems and sites, EC1 from Dal Lake and PF1 from soil samples of Ganderbal tolerated the maximum concentration of chlorpyrifos (11 mg/ml) and their minimum inhibitory concentration (MIC) was found to be 12 mg/ml (Table 1). The presence of highly CP<sup>r</sup>-tolerant bacterial isolates indicates that these areas are continuously being exposed to the toxic insult of chlorpyrifos. These results agree with earlier reports that indicate that due to excessive use of CP, a great diversity can exist in CP-resistant bacterial isolates in water and soil ecosystems of Kashmir valley<sup>11</sup>.

The organophosphate-degrading genes (opd gene) isolated from various species have been found to be present

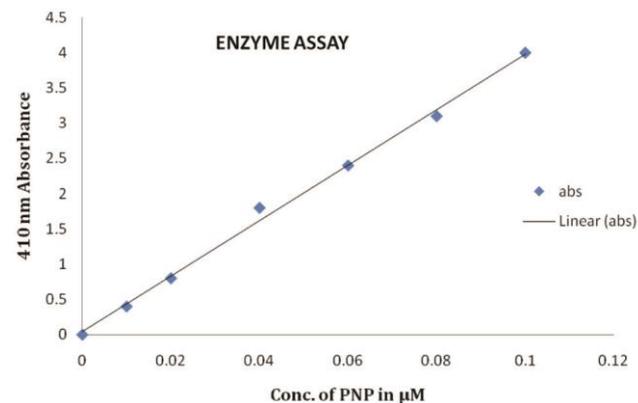
either on plasmid or located on the chromosome<sup>21,22</sup>. To determine location of chlorpyrifos resistance determinant in wild EC1 and PF1 isolates, plasmid curing experiments were performed. Our results revealed that while using 150 µg/ml of acridine orange as curing agent, the cured wild PF1 isolate was unable to show any visible colony on the plates amended with 2 mg/ml chlorpyrifos stress as compared to chlorpyrifos free plates that demonstrated well isolated colonies (data not shown). Interestingly, in case of EC1 isolate, acridine orange treatment did not show any visible effect as the acridine orange-treated cells were able to show normal growth on chlorpyrifos amended plates (5 mg/ml). As PF1 isolate could not even grow on lowest chlorpyrifos stress (0.5 mg/ml) after treating with acridine orange, there is strong evidence that in this cured isolate, plasmids possess genetic determinant involved in degradation of chlorpyrifos. To reconfirm these results, transformation studies with wild plasmids of EC1 and PF1 were carried out in DH5α competent cells and the location of chlorpyrifos resistance in PF1 *P. fluorescens* was authenticated to be plasmid born but not in EC1 *E. coli* and reference strain MTCC-533. It is clear that PF1 plasmid from the wild bacterial isolate of *P. fluorescens* was able to transform chlorpyrifos sensitive DH5α cell into chlorpyrifos-resistant cells that were able to demonstrate almost the same CP resistance pattern as shown by wild type *P. fluorescens* (data not shown).

The microbial bioremediation of CP mediated via hydrolytic and oxidative enzymes in soil and aquatic environments plays an important role in its degradation. The biodegradation of such compounds by bacterial key enzyme OPP has been reported in many microorganisms<sup>23</sup>. In order to check the biochemical mode of action of chlorpyrifos degradation in the selected EC1 and PF1 bacterial isolates, OPP enzymatic assay was performed that revealed presence of OPP mode of chlorpyrifos degradation present in each isolate. As reported in earlier studies also, as compared to intracellular activity, the

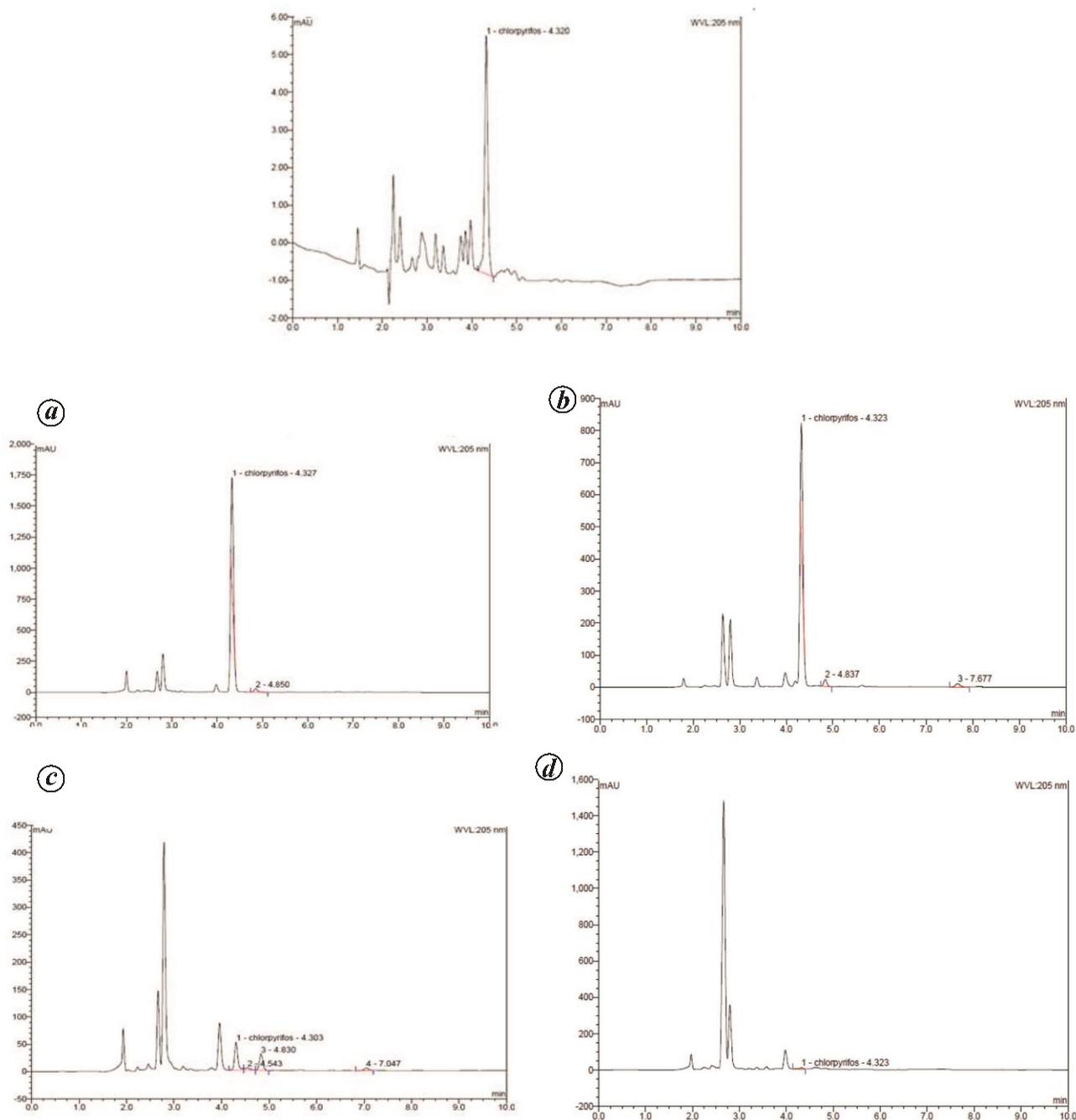
**Table 1.** Chlorpyrifos resistance pattern of selected isolates

Isolate	Chlorpyrifos stress (mg/ml)							
	8	9	10	11	12	13	14	15
EC1	+	+	+	+	–	–	–	–
EC2	+	+	+	–	–	–	–	–
EC3	+	–	–	–	–	–	–	–
EC4	+	+	–	–	–	–	–	–
EC5	+	+	+	–	–	–	–	–
P.F1	+	+	+	+	–	–	–	–
P.F2	+	–	–	–	–	–	–	–
PF3	+	+	+	–	–	–	–	–
PF4	+	+	–	–	–	–	–	–
PF5	+	–	–	–	–	–	–	–

+ represents growth on chlorpyrifos amended LB agar plates.



**Figure 1.** Standard graph of PNP curve of OPP enzyme liberating 1 µM *p*-nitrophenol per minute at 37°C.



**Figure 2.** Chromatogram of standard chlorpyrifos. *a*, Chromatogram indicating degradation of CP (2 mg/ml) after 7 days in absence of bacteria (Negative control). *b*, Chromatogram of chlorpyrifos degradation in presence of MTCC-533 reference strain *E. coli* at 2 mg/ml CP (positive control). *c*, Chromatogram of chlorpyrifos degradation in presence of *E. coli* (EC1) at 2 mg/ml CP. *d*, Chromatogram of chlorpyrifos degradation in presence of *P. fluorescens* (PF1) at 2 mg/ml CP chromatogram.

OPP enzyme activity was found to be present slightly more in extracellular fraction in both EC1 (0.060) and PF1 (0.058) isolates (Figure 1, Tables 2 and 3).

Various bacterial species that have been reported to grow on chlorpyrifos are capable of utilizing CP as the sole source of carbon, phosphorus and energy and thus

degrading it into its simpler constituents. Latifi *et al.*<sup>24</sup>, while using high performance liquid chromatographic (HPLC-UV) based analysis reported that some bacterial species inhabiting water and soil ecosystems are able to degrade chlorpyrifos in a time-dependant manner. In the current study, EC1 and PF1 selected isolates tolerated

5 mg/ml and 7 mg/ml of chlorpyrifos in minimal media respectively (data not shown). Therefore, for HPLC based CP degradation analysis, in this study, only two concentrations, viz. 2 mg/ml and 5 mg/ml of CP were used and retention time of standard chlorpyrifos was established to be 4.32 minutes (Figure 2). The present study revealed that biodegradation of chlorpyrifos continued gradually throughout the experiment till the termination of incubation on the 7th day. We also found that biodegradation of chlorpyrifos by both EC1 and PF1 is a growth-linked process, as also reported recently by Chisti *et al.*<sup>25</sup> in *Agrobacterium* and *Enterobacter* isolates collected from soil and water ecosystems. Interestingly, it was found that both the selected wild isolates were able to degrade chlorpyrifos far more efficiently than the well characterized *E. coli* MTCC-533 reference strain used in this study and the soil bacteria PF1 proved to be the most efficient isolate in degrading CP (Figure 2 a-c). The reference *E. coli* MTCC-533 strain comparatively showed lesser degradation of CP at 2 mg/ml stress level (60%) as well as at 5 mg/ml (0.5%) stress levels (Table 4). The water-borne EC1 isolate was able to degrade 68% CP at 2 mg/ml stress level but only 5.32% at 5 mg/ml CP stress level in the minimal media within 7 days of experiment. The result indicates that at higher CP concentrations, in case of EC1, there is a drastic decrease in degradation capability. At 2 mg/ml stress level, PF1 iso-

late demonstrated significantly higher level of chlorpyrifos degradation (85%) than EC1. Interestingly, at 5 mg/ml stress level there existed a great variation among them with PF1 showing almost 8 times more degradation (8%) capability than EC1. Harishankar *et al.*<sup>5</sup> while using almost 2000 times lesser stress levels of CP (0.1 mg/ml) than those used in our study, reported that the intestinal *E. coli* isolates were able to degrade only 16% of CP after 15 days incubation, which is almost 50 times lesser than that demonstrated by wild EC1 isolates. Therefore, from our HPLC based CP degradation analysis it is clear that the two selected bacterial isolates (EC1 and PF1) are much more efficient and can be employed effectively for degradation of heavily CP polluted water and soil ecosystems in the area.

Pesticides, like CP, currently being used in abundance has shown its presence both in water bodies as well as in soil ecosystems of Kashmir valley and is thus a cause of major concern. Successful removal of pesticides by the addition of bacteria was reported earlier for many pesticides. The present study reports isolation and biochemical and molecular characterization of CP-resistant wild soil and water-borne bacteria. The HPLC based results suggest that both the selected bacterial isolates degrade higher levels of chlorpyrifos. Interestingly in *Pseudomonas fluorescens* PF1 isolate, plasmid-borne genetic determinants were responsible for degradation of chlorpyrifos. The study revealed that there exists a large number of CP-resistant bacteria isolates in the natural environments of Kashmir valley. The results confirm that the presence of highly efficient chlorpyrifos-degrading bacterial isolates like EC1 (*E. Coli*) and PF1 (*Pseudomonas fluorescens*) present in different ecosystem of Kashmir Valley could be successfully exploited for bioremediation of CP polluted soil and water eco-systems of the area.

**Conflict of Interest:** There exists no conflict of interest among the authors.

**Table 2.** Enzyme activity in extracellular fractions

Strain	OPP activity
E.C 1	0.060
P.F 1	0.058
MTCC <i>E. coli</i> (ref. strain)	0.055

**Table 3.** Enzyme activity in Intracellular fractions

Strain	Activity
E.C 1	0.058
P.F 1	0.057
MTCC <i>E. coli</i> (ref. strain)	0.055

**Table 4.** Degradation pattern of chlorpyrifos after 7 days by bacterial isolates used in the present study

Isolates	Concentration	
	Degradation at 2 mg/ml stress level	Degradation at 5 mg/ml stress level
<i>E. coli</i> (EC 1)	68%	1%
<i>P. fluorescens</i> (PF1)	85%	8%
MTCC <i>E. coli</i> (Ref. Strain)	60.0%	0.5%

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## An improved microscopic method for the rapid diagnosis of emerging microsporidian parasite, *Enterocytozoon hepatopenaei* in shrimp farms

P. Ezhil Praveena, T. Bhuvaneshwari, A. Navaneeth Krishnan, V. Jagadeesan, J. Joseph Sahaya Rajan and K. P. Jithendran\*

Aquatic Animal Health and Environment Division, ICAR-Central Institute of Brackishwater Aquaculture, 75, Santhome High Road, R.A. Puram, Chennai 600 028, India

***Penaeus (Litopenaeus) vannamei* shrimp samples (hepatopancreas and faeces) collected from grow-out farms were evaluated for the presence of newly emerged microsporidian parasite *Enterocytozoon hepatopenaei* by concentration techniques using either water–ether sedimentation or Sheather’s sugar solution for spore recovery and subsequent microscopic detection by modified trichrome stain (Ryan-blue method). This improved selective staining protocol can easily resolve the identification of microsporidian spores in hepatopancreatic tubules compared to conventional stains. This method enables differential diagnosis of microsporidian spores by a characteristic staining pattern of pinkish-red, often with a belt-like diagonal stripe seen in the middle of the spore and a halo of unstained area at one end which could be easily distinguished from similarly staining particles or debris.**

**Keywords:** *Enterocytozoon hepatopenaei*, microsporidia, trichrome staining, shrimp aquaculture.

MICROSPORIDIA are obligate, intracellular, spore-forming endoparasites known to infect a wide range of eukaryotic hosts, both terrestrial and aquatic. To date, approximately

\*For correspondence. (e-mail: kpjithendran@ciba.res.in)