GROWTH PATTERN AND HERBICIDE UTILIZATION PROFILE OF ATRAZINE AND ANILOFOS RESISTANT AUTOCHTHONOUS BACTERIAL ISOLATES

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ABSTRACT

Total heterotrophic bacterial population (THBP) and atrazine & anilofos (herbicides) resistant bacterial population in a cultivated field was enumerated. The herbicide resistant bacterial isolates were characterized and identified up to genus level. The THBP in the field samples (water and sediment) are in the range of $22-71 \times 10^6$ CFU ml$^{-1}$ and $80-100 \times 10^6$ CFU gm$^{-1}$ respectively. The atrazine resistant bacterial population in sediment samples was $22-72 \times 10^6$ CFU gm$^{-1}$ and $18-67 \times 10^3$ CFU ml$^{-1}$ in water samples. Similar results are reported on anilofos resistant bacterial load. Herbicide resistant bacterial population was composed of Bacillus sp. (38.81%), Pseudomonas sp. (25.50%), Proteus sp. (21.4%) and Micrococcus sp. (14.0%). All the four bacterial isolates were found to utilize both the herbicides (atrazine and anilofos) up to 0.1% (Al) concentration of the herbicides. Among the herbicide utilizing bacterial isolates, Bacillus sp. was found to utilize both atrazine and anilofos efficiently with superior growth profile.

KEYWORDS: Herbicide, Atrazine, Anilofos, Biodegradation, Pseudomonas, Bacillus.

INTRODUCTION

Atrazine (6 - chloro - N$^2$ - ethyl - N$^4$- isopropylaminino 1, 3, 5 - triazine 2, 4 diamine) is a selective pre- and post- emergent herbicide used in paraguay, forestry, grassland, crops, maize, sugarcane etc. Anilofos (s - 4 chloro - N - isopropyl - benzoyl methyl - O, O - dimethyl phosphonodi- iate) is a herbicide effective against annual aminaceous weeds and sledges. Both these herbicides are commonly used in crop and sugarcane fields. These farm chemicals are degraded sparingly by natural mechanisms (physico-chemical) and were found to persist in soil and water ecosystem (Sankaran et al., 1993). With to their toxicity, the residual pesticide and its metabolites pose serious threat to the environment and are indeed a cause of concern (Deo et al., 1994). The non-target effect of these herbicides especially on beneficial microbial population is well-documented (Chakaravarthy and Chaterpaul, 1989). Use of microorganisms, for the removal of environmental pollutants is an obvious choice, as microorganisms play an important role in the transformation of many of these farm chemicals in nature. In fact, one environment management strategy includes process to stimulate appropriate microbial activities by supplying nutrients (Havel and Reineke, 1992).

In view of excess and continuous use of these herbicides, it is imminent to employ appropriate bioremediation strategies to restore the soil fertility. This paper reports the distribution of

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herbicide resistant bacteria in a cultivated field and their growth pattern with the chosen herbicides as the sole carbon source.

MATERIALS AND METHODS

Kadana river, originating in the Western Ghats, is harnessed to irrigate crop fields in Alwarkurichi, village, panchayat, Ambasamudram Taluk, Kurnool District, Tamil Nadu. Samples were drawn from a sugarcane field. Sediment samples were collected from 4 corners and central area of the field, pooled in a gamma irradiated polythene bag and transported to the laboratory in an ice pack. Water samples from irrigation channel inlet and outlet of the field were collected in sterile Erlenmeyer flask (250 ml), transported in an ice pack and processed within 6 hours of collection. Sampling was carried out for 5 cycles over a period of 8 months.

Numeration of total heterotrophic bacterial load

Sediment and water samples were decimally diluted with sterile distilled water blanks and dilutions $10^4$, $10^7$ and $10^8$ were used as inoculum. The plating technique was employed using sterile nutrient agar media (Himedia, Mumbai) and the plates were incubated at 37°C for 24-48 hrs.

Numeration of herbicide resistant bacterial load

Herbicides, atrazine and anilofos were incorporated in sterile molten nutrient agar medium (40-45°C) at different concentrations of Active Ingredient (AI) (1.0%, 0.1% and 0.01%) before solidification. Sediment and water samples were serially diluted with sterile distilled water and 0.1 ml of dilutions $10^3$, $10^4$ and $10^5$ were spread plated on air-dried herbicide incorporated nutrient agar plates and incubated at 37°C for 24-48 h. Morphologically similar colonies were pure cultured by quadrant streaking technique and stored in nutrient agar slants.

Characterization of Herbicide Resistant Bacterial (HRB) isolates

The HRB isolates were characterized up to generic level on the basis of their morphological, biochemical and physiological characteristics adopting the methodology recommended by Simudo and Aiso (1962).

Evaluation of herbicide utilization by HRB isolates

This was carried out adopting the methodology of Walker et al., (1993). Mineral salt medium ($KHP_4$O$_4$-1.0 g, $K_2HPO_4$ - 1.0 g, $NH_4NO_3$ - 1.0 g, $MgSO_4$ - 1.0 g, $CaCl_2$ - 0.02 g, $FeSO_4$ - 0.01 g, Distilled water - 1000 ml, pH - 6.5) was prepared and autoclaved. After cooling this to 45°C, atrazine or anilofos was incorporated at different concentration of AI (1.0%, 0.1% and 0.01%) as a sole carbon source and dispensed into petri plates. Overnight nutrient broth cultures of HRB isolates were centrifuged at 10,000 rpm for 10 minutes at room temperature in a table top centrifuge (Remi C-24) and washed twice with sterile saline (0.85% NaCl) and then resuspended in 1.0 ml sterile saline. These saline suspensions of HRB isolates were inoculated on air dried herbicide incorporated MSM plates by employing single streak method and incubated at 37°C for 24-48 h.

Growth studies of herbicide utilizing bacterial isolates

The herbicide utilizing bacterial isolate was sub cultured in sterile nutrient broth (10 ml). These overnight cultures were centrifuged at 10,000 rpm for 10 minutes and washed twice with sterile saline (0.85% NaCl) and resuspended in the same. This saline suspension of the isolate was inoculated into atrazine/anilofos (0.01%, AI) incorporated sterile MSM broth at 10% concentration (v/v) and incubated at 37°C for 48-72 h. with agitation for acclimatization of bacterial isolates to test chemicals. These acclimatized test culture was inoculated into 100 ml MSM with atrazine/anilofos (0.1%, AI) at the concentration of 10% (v/v) in a 250 ml Erlenmeyer flask and incubated at 37°C with agitation (120 rpm) in an orbital shaker (Neolab). At fourth hourly interval, samples were withdrawn from the broth culture, aseptically and the optical density (OD) was recorded in a spectrophotometer (Spectronic 20D) at 530 nm. The sampling time (X-axis) against OD$_{530}$ (Y-axis) was plotted to deduce the growth pattern of the bacterial
Table 1. Total heterotrophic and herbicide resistant bacterial population in the sediment and water samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total heterotrophic bacterial population ( \times 10^6 \text{CFU/gm/ml} )</th>
<th>Total Atrazine resistant bacterial population ( \times 10^6 \text{CFU/gm/ml} )</th>
<th>Total anilofos resistant bacterial population ( \times 10^6 \text{CFU/gm/ml} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>80–100</td>
<td>1.0%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Irrigation channel inlet water</td>
<td>39–71</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Irrigation channel outlet water</td>
<td>22–40</td>
<td>1.0%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

Water and sediment samples were withdrawn from a crop field and screened for total heterotrophic (THBP) and herbicide resistant bacterial population (HRBP). The THBP was found to be in the range of 22–100 \( \times 10^6 \text{CFU/gm} \). The RBP was in the order of 10^1 CFU ml^-1/gm for both atrazine and anilofos (Table 1). The herbicide resistance exhibited was observed to be concentration dependent as atrazine and anilofos were inhibitory to bacterial isolates at higher concentrations (1.0%, 0.1%). The herbicide resistant bacterial population is composed of four genera namely, *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., and *Proteus* sp. Among these, *Bacillus* sp. (38.8%) was the dominant strain followed by *Pseudomonas* sp. (25.5%) (Fig. 1). The native bacterial isolates were designated as *Bacillus* SPK 05, *Pseudomonas* SPK 10, *P. aeruginosa* SPK 03 and *Micrococcus* SPK 07. Even though pesticide resistant *Bacillus*, *Pseudomonas* and *Micrococcus* were frequently reported by Yamada and Kuwatsuka, 1990; Topp et al. 1992; Roberts et al. 1993), presence of herbicide resistant *Proteus* sp. was not reported earlier. The presence of *Pseudomonas* sp. could be attributed to the contamination of irrigation water channel with local sewage and so because of this water being used for bathing, shaving and other domestic purposes by the local public.

All the four bacterial isolates were observed to utilize both atrazine and anilofos as the sole carbon source at the concentrations of 0.1% and 0.01% (AI) of the herbicides which is evident from the plate count assay (Table 2). Among the herbicide utilizing bacterial isolates, *Bacillus* SPK 0.5 was found to be efficient in utilizing atrazine (0.1% AI) as the sole carbon source, followed by *Pseudomonas* SPK 10 (Fig. 2). Effective mineralization of a wide range of pesticides by *Bacillus* sp. was reported by Sahu et al., 1993), *Pseudomonas* sp. by Zboinska et al., 1992) and *Micrococcus* sp. by Bevinakatti and Ninnekar (1993). Similar pattern of utilization was observed with anilofos as the sole carbon source (Fig 3). Such
Table 2. Plate count assay for herbicide utilization by the bacterial isolates

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Atrazine (Conc., Al)</th>
<th>Anilofos (Conc., Al)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0%</td>
<td>0.1%</td>
</tr>
<tr>
<td><em>Escherichia</em> SPK 05</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><em>Pseudomonas</em> SPK 10</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><em>Aeromonas</em> SPK 01</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td><em>Morococcus</em> SPK 03</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ = Luxuriant growth; ++ = Moderate growth; - = Absence of growth.

Fig. 2. Growth response pattern of herbicide utilizing bacterial isolates with atrazine (0.1%, A.I) as the sole C-source.

Fig. 3. Growth response pattern of herbicide utilizing bacterial isolates with anilofos (0.1%, A.I) as the sole C-source.

Our investigation reveals the presence of a relatively low population of herbicide resistant/lizing bacteria in the crop field which was restricted to four bacterial genera. Their sensitivity to higher concentrations (1.0% Al) of the commonly used herbicides namely, atrazine and anilofos is indeed a cause of concern. As these herbicides are used continuously due to aggressive cropping, a slow increase in residual concentration of these farm chemicals may result in a drastic reduction in the native microflora, which includes herbicide resistant bacteria. Hence, it becomes absolutely necessary to monitor the residual concentration of herbicides and their metabolites periodically and design comprehensive strategies to prevent the phenomenon of bioaccumulation of these farm chemicals in soil ecosystem. Since the autochthonous bacterial isolates were able to utilize these herbicides as sole carbon source, further investigations on the biochemical basis of degradation, their interaction as a consortia and scope of manipulation of their genetic system for improved herbicide degradation would prove invaluable in employing them for bioremediation of atrazine and anilofos polluted soil and water ecosystem.

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facilities to carry out this study. The authors also wish to thank Dr. Natesan, Rallis India Ltd., Bangalore for providing herbicide samples and standards.

REFERENCES


DISTRIBUTION OF BUTACHLOR METABOLISING BACTERIA IN A PADDY FIELD

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Key words: Butachlor, bio-remediation, degradation, Pseudomonas sp., Micrococcus sp., Bacillus sp. and Proteus sp.

ABSTRACT

The total heterotrophic and butachlor resistant bacterial population in a paddy field was enumerated. Soil and irrigation water samples were drawn from a cultivated paddy field analysed for Total Heterotrophic Bacterial Population (THBP) and Total Butachlor Resistant Bacterial Population (TBRBP). THBP was in the range of 60 x 10^3 to 12.6 x 10^7 CFU/gm of soil and 20-75 x 10^3 CFU/ml of irrigation water. The TBRBP was found to be 58-73 x 10^3 CFU/gm and 70 - 88 x 10^3 CFU/ml in soil and water respectively. Butachlor resistant bacterial isolates were identified and characterized up to generic level. The butachlor resistant isolates were screened for their butachlor metabolising capacity with different concentrations of the herbicide (butachlor). Among the butachlor resistant bacterial isolates Bacillus sp. (58%) was predominant followed by Pseudomonas sp (24%) and Micrococcus sp (10%). We also report the incidence of butachlor resistant Proteus sp. All four bacterial genera were found to utilise butachlor as the sole carbon source at 0.1% concentration. Micrococcus sp was the most efficient butachlor utilising bacterium followed by Proteus sp. and Bacillus sp.

INTRODUCTION

Butachlor (N-butoxy methyl - 2, 6' diethyl acetanilide) is a most frequently used herbicide in the paddy field. It is a pre-emergent herbicide for the control of annual grasses and certain broad leaved weeds in rice. On application, butachlor persists for 6-8 weeks and its residues for longer duration. Degradation of butachlor by physico-chemical factors is negligible (Sankarathica!, 1993). As it is known, microorganisms play a pivotal role in the transformations of many soil applied farm chemicals. Presence and persistence of butachlor degrading microorganisms would play a vital role in its degradation and clearance from the paddy field (Comeau et al., 1993; Roberts et al., 1993). In view of the inherent toxicity and non-target effects of herbicides it would be appropriate to understand the degradation abilities and patterns of the native bacterial communities which would prove invaluable in designing bio-remedial measures (Deo et al., 1994).

This study was taken up to screen a paddy field soil and irrigation water for butachlor resistant bacteria. Further, the bacterial isolates were characterized. Growth studies with butachlor as a sole carbon source were performed to evaluate the butachlor utilising efficiency of the isolates.

MATERIALS AND METHODS

Soil samples were collected from a cultivated paddy field at Alwarkurichi, a town Panchayat situated in the Tirunelveli District. The samples were collected in pre sterilised polythene bags. Simultaneously, irrigation water was collected in a sterile Erlenmeyer flask (250 ml) at points of entry into the field and exit. The soil and water samples were transported to the laboratory in an ice pack. Sampling was carried out for 6 months at monthly intervals.

Enumeration of Total Heterotrophic Bacterial Population (THBP)

The soil and water samples were decimally diluted with
sterile glass distilled water up to 10³. One ml aliquots of appropriate dilutions were poured into sterile petri plate and 15-20 ml of molten sterile nutrient agar (Hi-Media, Mumbai) was added into the petri plate. The inoculated plates were incubated at 37°C for 48 hours. Petriplates containing 30-300 colonies were selected for the enumeration of THBP.

**Enumeration of total Butachlor Resistant Bacterial Population**

The herbicide, butachlor was filter sterilised and incorporated into the molten sterile nutrient agar medium at different concentrations of active ingredient (0.01%, 0.1% and 1.0%). The enumeration of butachlor resistant bacteria was carried out as per the methodology adopted for the enumeration of THBP.

**Identification and characterisation of Herbicide Resistant Bacterial Population**

From the butachlor incorporated plates, morphologically dissimilar colonies were streaked on sterile nutrient agar plates for purity. The pure bacterial isolates were restreaked in nutrient agar slants and stored at 4°C to maintain the viability of the bacterial strains. The isolates were identified as per the standard procedures (Simudu and Aiso, 1962, Benson, 1990). The Bergey’s manual of Determinative Bacteriology was also referred in the identification procedure.

**Butachlor utilisation by the bacterial isolates - Plate count assay**

Filter sterilised butachlor was incorporated into sterile mineral salt agar medium (Walker et al, 1993) at different concentrations of active ingredients (A.1) (0.01%, 0.1% and 1.0%). On these air dried, butachlor incorporated plates, 0.1 ml of fresh butachlor resistant bacterial isolates were spread and incubated at 37°C for 24-48 hours. The initial load of the inoculum was determined through plate count method using sterile nutrient agar medium.

**Growth studies of butachlor utilising bacterial isolates**

The butachlor resistant bacterial isolates were inoculated into sterile mineral salt broth (Walker et al, 1993) with butachlor (0.01%, 0.1% and 1.0% A.1) and incubated in a water bath shaker for 24-48 hours at 37°C. Samples were withdrawn at 3 hours intervals and analysed photometrically. The growth pattern was deduced by plotting the optical density against sampling time.

### Table 1. Total heterotrophic and butachlor resistant bacterial load in the paddy field (Range of 6 Cycles)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Heterotrophic bacterial Load (X 10⁶ CFU/gm/ml)</th>
<th>Butachlor resistant bacterial Load (X 10⁶ CFU/gm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Butachlor 1.0%</td>
</tr>
<tr>
<td>Soil</td>
<td>60-126</td>
<td>-</td>
</tr>
<tr>
<td>Inlet water</td>
<td>20-35</td>
<td>-</td>
</tr>
<tr>
<td>Outlet water</td>
<td>44-75</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 2. Plate count assay for butachlor utilisation by the paddy field bacterial isolates

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Initial bacterial load (CFU/ml)</th>
<th>Final bacterial load (X 10⁶ CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butachlor 1.0%</td>
<td>Conc. 0.1% (A.1) 0.01%</td>
<td></td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>165 × 10⁶</td>
<td>51.0</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>130 × 10⁶</td>
<td>40.0</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>128 × 10⁶</td>
<td>35.0</td>
</tr>
<tr>
<td>Micrococcus sp.</td>
<td>156 × 10⁶</td>
<td>60.0</td>
</tr>
</tbody>
</table>
RESULTS

Soil heterotrophic bacterial population (THBP) was in the order of $60 \times 10^{2}$ to $12.6 \times 10^{4}$ CFU/gm. The THBP in inlet and outlet water was $20-35 \times 10^{4}$ CFU/ml and $75 - 10^{5}$ CFU/ml respectively (Table 1). The butachlor resistant bacterial population (TBRBP) of soil was $58-70 \times 10^{3}$ CFU/gm and $87-109 \times 10^{3}$ CFU/gm at 0.1% and 0.01% of butachlor (A.I) respectively. The TBRBP in the outlet water was $70 - 88 \times 10^{4}$ CFU/ml and $90 - 101 \times 10^{4}$ CFU/ml at 0.1% and 0.01% butachlor concentration (A.I) respectively. No bacterial growth was observed at higher butachlor concentration 1.0% A.I (Table 1).

Among the butachlor resistant bacteria, Bacillus sp. (8%) was predominant followed by Pseudomonas sp. (4%) and Micrococcus sp. (10%) and Proteus sp. (6%) (Fig. 1).

All four butachlor resistant bacterial genera were found to utilise butachlor when inoculated on butachlor supplemented MSM agar (0.01% and 0.1% of A.I) (Table 2). Growth studies in butachlor supplemented SM broth showed differential growth pattern among the butachlor resistant bacterial genera. Micrococcus exhibited maximum butachlor utilisation on the basis of growth rate followed by Bacillus sp. and Proteus sp. (Fig. 2). The utilisation of butachlor as a sole carbon source by Pseudomonas sp. was comparatively low.

DISCUSSION

The total heterotrophic bacterial population and the butachlor resistant bacterial population in the paddy field were in the range of $10^{3} - 10^{4}$ CFU/gm/ml of soil and irrigation water samples (Table 1 and 2). The above findings clearly indicated that, the native heterotrophic bacterial population of soil and irrigational water seemed to be comprised primarily of butachlor resistant bacterial population. This could be due to the selective enrichment of herbicide resistant bacterial population on repeated application of butachlor in the soil (Walker et al., 1993). The present investigation also revealed that the butachlor resistant bacterial population were unable to tolerate the butachlor at concentrations above 0.1% (A.I). Hence, accumulation of these herbicides and their residues in the soil due to continuous application could jeopardise the survival of even herbicide resistant/degrading bacteria, which may have serious implications on the soil fertility. Such impact of pesticides on soil microorganisms was reported by Brunsbach and Reineke (1993) with aroclor.

As Bacillus sp. and Pseudomonas sp. are the most common native microflora in the soil environment, their higher incidence in the paddy field are well anticipated (Fig. 1). Such lines of inference can be drawn on the occurrence of Micrococcus sp. also. The incidence of butachlor resistant Proteus sp. was not reported earlier.

Eventhough all 4 butachlor resistant bacterial isolates were found to grow in butachlor amended MSM plates, a distinctly different growth pattern was recorded in shake cultures. When analysing the growth pattern (Fig. 2) of the butachlor resistant organisms with butachlor as the sole carbon source in shake cultures Micrococcus sp. exhibited superior growth rate followed by Bacillus sp. and Proteus sp. Poor butachlor utilisation by Pseudomonas sp. was noticed. Efficient butachlor utilisation by Micrococcus sp and Bacillus sp can be attributed to their metabolic versatility and greater degree of adaptation. These two organisms were known to transform a wide
range of farm chemicals and other toxicants (Bevinakatti and Ninnekar, 1992; Zboinska et al., 1992).

Our study suggests the probable onset of selective enrichment of butachlor resistant microorganisms in the paddy field in response to the continuous use of this agrochemical. Even though such selective enrichment of herbicide (butachlor) degrading bacterial population hastens the disappearance of soil applied chemicals, concomitant reduction in the heterotrophic bacterial population including the beneficial microorganisms require closer monitoring. Further, in vitro studies on the degradation of butachlor, using butachlor utilising bacterial isolates would prove beneficial while formulating bio-remediation strategies to counter butachlor accumulation.

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The authors wish to thank the Management, Secretary and the Principal of Sri Paramakalyani College, Alwarkurichi, for providing necessary facilities to carry out this study.

REFERENCES


ABSTRACT: The pesticide (dimethoate) metabolising bacterial populations were isolated from the water and sediment samples collected from rice soil. Using standard methods, the isolates were identified and characterised. The total heterotrophic bacterial population in water and sediment samples ranged from $8.0 \times 10^4$ to $17 \times 10^4$ CFU/ml and $16.2 \times 10^4$ to $19.0 \times 10^4$ CFU/g respectively. The pesticide resistant bacterial load in the water and sediment samples varied between $2.7 \times 10^4$ to $5.7 \times 10^4$ CFU/ml and $3.3 \times 10^4$ to $8.6 \times 10^4$ CFU/g at 0.1% (active ingredient) pesticide concentration. Pesticide resistant bacterial genera primarily comprised of Bacillus, Pseudomonas, Micrococcus, Proteus and Flavobacterium. Growth studies were performed to evaluate the pesticide utilisation by these bacterial genera. Micrococcus sp and Proteus sp were found to be the most efficient in utilising pesticide dimethoate as the sole carbon source followed by Pseudomonas sp.

INTRODUCTION

After the introduction of D.D.T. in 1939 by Paul Müller, a number of compounds including cyclodienes appeared in the pesticide market in quick succession. Intensive cropping to meet the increasing demand has resulted in an increase in the number and types of pesticides used. With the increase in the use of pesticides there exists an increased risk of pesticide cumulation in the soil. It has been demonstrated that most of these farm chemicals have entered the ecosystem and increase in their concentration in the environment has led to the process of biomagnification. These pesticides contaminate the soil, water, dairy products and other commodities. Microorganisms are omnipresent and biochemically omnipotent. Over millions of years, microorganisms have evolved an extensive range of enzymes, pathways and control mechanisms to degrade an array of compounds. Even though farm chemicals are degraded meagerly by physical factors, owing to their versatility, microbial degradation of pesticides and their residues is the most favoured process to get over this problem of accumulation (Deo et al., 1994). Rogor (dimethoate 1%) is one of the most widely used broad spectrum pesticides in crops like paddy, cotton, chillies, groundnut etc.

Accumulation of pesticide and its residues in soil is well documented (Deo et al., 1994). In this context, the present study was taken up to screen the soil samples collected from the paddy field at Tenkasi Taluk, Tirunelveli District, Tamil Nadu for the isolation and identification of dimethoate resistant and utilising microorganisms. Further, the growth pattern of these bacterial isolates was also studied using dimethoate as the sole carbon source.

MATERIALS AND METHODS

Samples for this study were collected from a cultivated rice soil at Tenkasi Taluk, Tirunelveli District. Monthly samples were drawn over a period of six months and used in this study.

Samples

Soil and water samples from the paddy field were analysed in this study. Representative soil samples were collected from four corners and the centre of the paddy field mixed aseptically and used for various microbiological analysis. Water used for irrigation was collected at the point of entry and exit in the paddy field separately in a sterile Mac Cartney bottles and placed in an ice box and transported and microbiological analysis was performed within six h of collection.

Enumeration of total heterotrophic bacterial load

The soil and water samples were decimally diluted with sterile distilled water and dilutions of $10^{-1}$, $10^{-4}$ and $10^{-5}$ were used as inoculum for pour plating using nutrient agar medium (Peptone - 0.5%, Beef extract - 0.3%, NaCl - 0.5%, and Agar - 1.5% pH 7.0).

Enumeration of pesticide resistant bacterial load

Pesticide dimethoate was incorporated in the nutrient agar medium at various concentrations (1.0%, 0.1% and 0.01% of active ingredient). Soil and water samples were serially diluted and 1ml of the chosen dilution was inoculated in the nutrient agar plates.
incorporated with pesticide. The inoculated plates were incubated at 37°C +/- 0.1 for 48 - 72 h.

**Identification of pesticide resistant bacteria**

The bacterial isolates grown in the pesticide resistant plates were restreaked on nutrient agar plates to get pure cultures and the bacterial strains were identified based on their morphological, physiological and biochemical characteristics up to generic level using standard identification procedures (Aneja, 1993;enson, 1990).

**Evaluation of bacterial isolates for pesticide utilisation**

Pesticide resistant isolates were sub cultured overnight in sterile nutrient broth (Peptone - 0.5%, NaCl - 0.5% and Beef extract - 0.3%, pH 7.0) and serially diluted. Appropriate dilutions were poured plated in mineral salt agar plates (KH2PO4 - 0.1%, KH2PO4 - 0.1%, NH4NO3 - 0.1%, MgSO4 - 0.1%, CaCl2 - 0.002%, FeSO4(3-0.001% and agar 1.5%, pH 6.5) incorporated with dimethoate (1.0%, 0.1% and 0.01% active ingredient). The initial load of the test isolate was also recorded using nutrient agar medium.

**Growth studies of pesticide utilising bacterial isolates**

The growth pattern of pesticide utilising bacteria was studied by the known method (Walker et al., 1993). One ml of overnight culture of dimethoate resistant bacteria was inoculated into the mineral salt solution containing different concentrations of dimethoate and incubated in a water bath shaker. The growth response of the isolates to pesticide was observed colorimetrically at 580 nm using spectronic-20 by recording the optical density every 2 h for 48 h.

**RESULTS AND DISCUSSION**

The heterotrophic bacterial population of soil and water samples of paddy field ranged from 162 - 190 X 10⁶ CFU/g and 8 - 17 X 10⁸ CFU/ml respectively (Table 1). Majority of the heterotrophic bacterial population seems to be pesticide resistant as the dimethoate resistant bacterial population at concentrations 0.1% and 0.01%, is almost similar to that of the total heterotrophic bacterial populations (Table 2). There was no bacterial growth observed in nutrient agar plates incorporated with higher concentrations of dimethoate (1.0%).

The dimethoate resistant bacterial population was restricted to five genera namely Pseudomonas, Bacillus, Micrococcus, Proteus and Flavobacterium. Among them, Pseudomonas sp. was found to predominate (42.0%) followed by Bacillus sp. (27.0%) and Micrococcus sp. (23.2%); other dimethoate resistant bacterial strains were Flavobacterium sp. (4.8%) and Proteus sp. (3.0%) (Table 3). Presence and persistence of pesticide resistant Pseudomonas sp. and Bacillus sp. was registered by many workers (Chatterjee et al., 1981, Crawford et al., 1979 Basavaraj and Harichandra, 1992). Topp et al., (1992) had reported the occurrence of pesticide resistant Micrococcus sp. and Flavobacterium sp. in sediment samples. The incidence of pesticide resisting and utilising Proteus sp. has not been reported earlier.

All the dimethoate resistant bacterial genera such as Pseudomonas, Bacillus, Micrococcus, Proteus and Flavobacterium isolated during the present study were also found to utilise the pesticide dimethoate at low concentration of 0.1% and 0.01% as the sole carbon source (Table 4).

When analysing the growth pattern of the five pesticide utilizing isolates, Micrococcus sp. sp. in spite of its extended lag, was found to have better growth rate in pesticide amended mineral medium followed by Bacillus sp. and Pseudomonas sp. Bauguin and Kiefer (1975) in their extensive trials with allachior have reported similar growth responses of Bacillus sp., Pseudomonas sp. and Micrococcus sp.

**CONCLUSION**

The predominance of pesticide resistant bacteria among the heterotrophic bacterial population suggests strongly of the possible onset of unpleasant phenomenon of bioaccumulation of pesticides. The presence of pesticide utilising and degrading microorganisms would be valuable in restoring soil fertility by degrading the pesticides making it possible for the survival of native and beneficial microflora. Hence, more research should be pursued in understanding the metabolism and degradation pathways of xenobiotics by microorganisms and their co-habitance with other beneficial microflora. So that the microbial diversity is conserved for the sustainable crop production.

**ACKNOWLEDGEMENT**

We wish to thank the Management, Secretary and the Principal of Sri Paramakalyani College, Alwarkuruchi, for providing us the necessary facilities to carry out the study.

**REFERENCES**


### Table 1. Total heterotrophic bacterial population of the cultivated field

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacterial load x 10^4 CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil (per g)</td>
<td>162 - 190</td>
</tr>
<tr>
<td>Inlet water (per ml)</td>
<td>8 - 12.</td>
</tr>
<tr>
<td>Outlet water (per ml)</td>
<td>14 - 17.</td>
</tr>
</tbody>
</table>

### Table 2. Dimethoate resistant bacterial population in the paddy field

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pesticide concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>Soil (per g)</td>
<td>-</td>
</tr>
<tr>
<td>Inlet water (per ml)</td>
<td>-</td>
</tr>
<tr>
<td>Outlet water (per ml)</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3. Percentage distribution of pesticide resistant bacterial isolates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>42.0</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.</td>
<td>27.0</td>
</tr>
<tr>
<td><em>Micrococcus</em> sp.</td>
<td>23.2</td>
</tr>
<tr>
<td><em>Flavobacterium</em> sp.</td>
<td>4.8</td>
</tr>
<tr>
<td><em>Proteus</em> sp.</td>
<td>3.0</td>
</tr>
</tbody>
</table>
### Dimethoate utilisation of the isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Initial load x 10^5 cells/ml</th>
<th>Final load x 10^6 cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pesticide concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>Bacillus sp</td>
<td>55</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>61</td>
<td>–</td>
</tr>
<tr>
<td>Micrococcus sp</td>
<td>72</td>
<td>–</td>
</tr>
<tr>
<td>Proteus sp</td>
<td>47</td>
<td>–</td>
</tr>
<tr>
<td>Flavobacterium sp</td>
<td>60</td>
<td>–</td>
</tr>
</tbody>
</table>

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MICROBIOTA OF TEXTILE MILL EFFLUENT, TREATMENT AND EFFECT OF TREATED EFFLUENT ON PLANT GROWTH

R. GOWRISANKAR, R. PALANIAPPAN and S. PONPANDI

Post Graduate Department of Microbiology, Sri Paramakalyani College, Alwarkurichi-627 412, Tamil Nadu

Key words: Effluent, Effluent treatment, Cymopsis tetragonaloba, Pseudomonas aeruginosa, Xanthomonas sp., Rhizosphere

ABSTRACT

Physico-chemical and microbiological characteristics of a textile mill effluent was analysed and data are presented. The pH of the effluent was in alkaline side with BOD values (400–1000 mg/l) well above the stipulated limits. The microflora was meagre and restricted only to bacteria. The effluent was subjected to various treatments and evaluated for its ability to support the plant growth. The effect of treated textile mill effluent on the plant (Cymopsis tetragonaloba) growth was evaluated, on the basis of seed germination, flowering and fruiting. Diluted effluent at concentrations of 25% and 50% was found to exert positive impact on plant growth. There was also significant reduction in the rhizosphere microflora of the test plant when treated with effluent at higher concentrations.

INTRODUCTION

Textile industry earns a sizable foreign exchange for our country. But various chemicals and dyes used in bleaching, dying and other processes give rise to a potentially toxic effluent (Dolar et al 1972). Microorganisms play a pivotal role in degrading complex toxic organic compounds and make them available for biological cycle. Most of these effluents with a number of toxic metals and higher BOD values are discharged into the riverine system which affects the native microflora and makes it a burden on the environment. Industrial effluent after proper treatment are often
used for irrigation in dry areas. The textile mill effluent not only contain rich organic compounds that enhance the growth of plants but also have toxic materials (Lincoln, 1985).

Hence, it is imperative to understand the implications of the textile mill effluent on plant growth. The present study was carried out to understand the microbial community of textile mill effluent, to treat the effluent using various techniques, evaluate the effect of treated effluent on the growth of the plant *Cymopsis tetragonaloba* and its rhizosphere microflora.

**MATERIAL AND METHODS**

The effluent samples for this study were collected from the effluent discharge stream of Madura Coats Textile unit situated in Vickramasiningh puram near Ambasamudram, Tirunelveli Dist. Standard methods (APHA, 1978) were used for the collection, transportation and preservation of the effluent samples. Physico-chemical parameters, of the textile mill effluent were recorded. The bacterial density, actinomycetes, yeast and mold counts of textile mill effluent were enumerated by adopting standard microbiological techniques (APHA, 1978). Total heterotrophic bacterial population was enumerated using nutrient agar medium (Hi-Media, Bombay) and for the enumeration of yeast and mold count rose bengal agar (Hi-Media, Bombay) was used. Actinomycetes were enumerated using Actinomycetes isolation agar (Hi-Media, Bombay). The bacterial strains were identified using the Bergey's Manual of Systematic Bacteriology (1987). The effluent was treated by the process of dilution. For this, the effluent was diluted with sterile distilled water at various concentrations namely 25%, 50% and 75%. This diluted effluent was used to irrigate test plants namely, *Cymopsis tetragonaloba*. An undiluted effluent and sterile distilled water control was also maintained. Plant growth was monitored periodically on the basis of seed germination, humule appearance, shoot and root length, flowering and fruiting, etc., observed up to a period of 40 days. At the end of the experiment period rhizosphere soil was withdrawn from test plants exposed to different effluent concentration and rhizosphere microbial load was enumerated using nutrient agar medium (Hi-Media, Bombay) to understand the influence of effluent on rhizosphere microflora.

**RESULTS AND DISCUSSION**

The physico-chemical parameters of the textile mill effluent is given in Table 1 and its bacterial density is shown in Table 2. Effluent had objectionable amounts of BOD values (400-1000 mg/l) against ISI standards. Hence, their discharge in the riverine system requires proper treatment. The pH of the effluent is alkaline and has an unacceptable green to greyish green colour with heavy load of suspended particles.
TABLE 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Parameter</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colour</td>
<td>Green to greyish green</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>7.98 to 9.0</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>29.8 to 31.6</td>
</tr>
<tr>
<td></td>
<td>BOD (mg/l)</td>
<td>400 to 1000</td>
</tr>
</tbody>
</table>

TABLE 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Parameter</th>
<th>Microbial load (CFU x 10^2/ML)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total heterotrophic bacterial load</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Yeast</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>Mold</td>
<td>Nil</td>
</tr>
<tr>
<td>4</td>
<td>Actinomycetes</td>
<td>Nil</td>
</tr>
</tbody>
</table>

These results can be corroborated with that of Edwards et al (1981).

Two bacterial species namely, *Pseudomonas aeruginosa* and *Xanthomonas* sp are, the lone microbial survivors in this effluent and is devoid of fungi, yeast and actinomycets. The effect of diluted effluent on plant growth is presented in Table 3. The raw effluent exerted very high degree of inhabitation on cluster bean (*Cymopsis tetragonoloba*) germination. On the other hand, 25%, 50% and 75% of effluent had shown positive effect on the germination of the plant. This type of positive influence was observed in flowering and fruiting of the plant also. Among various dilutions 50% was found to have better influence on germination, plumule formation and leaf dimensions. On the other hand 25% effluent concentration was observed to impart overall beneficial influence on plant growth in terms of flower and fruit numbers etc. over the control. This kind of impact of effluent on plant growth could be attributed to the presence of toxic materials, excess or deficiency of micronutrients and also to its effect on soil porosity and aeration, especially when the concentration of the effluent was increased (Someshekar et al, 1984).

Interestingly, our study indicated the beneficial effects of effluents on plant growth in terms of root and shoot length in dilutions of 25% and 50%. This could be due to the dilution of inhibitory chemicals in the effluent which may bring about a derepressive action on the plant growth (Rajannan and Oblisamy, 1979).
TABLE 3
Effect of effluent at various dilutions on plant growth
(Cymopsis tetragonaloba)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameter</th>
<th>Effluent concentrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>Seed germination (%)</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>Plummule formation (%)</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>Shoot length* (cm)</td>
<td>23.4</td>
</tr>
<tr>
<td>4</td>
<td>Root length* (cm)</td>
<td>7.7</td>
</tr>
<tr>
<td>5</td>
<td>Total leaf number*</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Mean leaf length* (cm)</td>
<td>3.45</td>
</tr>
<tr>
<td>7</td>
<td>Mean leaf width* (cm)</td>
<td>2.37</td>
</tr>
<tr>
<td>8</td>
<td>Total number of flower*</td>
<td>36</td>
</tr>
<tr>
<td>9</td>
<td>Total number of fruits*</td>
<td>28</td>
</tr>
</tbody>
</table>

* - Mean value of eight seeds. b - Mean value of four plants.

Results presented in Table 4 indicates the inhibitory effect of effluent on rhizosphere microflora with the increase in the effluent concentration. This could also be a reason for the negative impact of effluent on plant growth in its higher concentration.

The present study confirms the adverse effects of textile mill effluent on the riverine system and on the growth of plants as well as native microflora. Nevertheless, suitable treatments such as dilution brings down its toxic effect and makes it less harmful to the environment. In fact the treated effluent was comparable with that of control in supporting plant growth at appropriate dilution.

Hence, it is suggested that the textile mill effluent on proper treatment such as dilution can be used for agricultural purposes.

TABLE 4
Effect of treated effluent on the Rhizosphere microflora

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Micro organisms</th>
<th>Effluent concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>Bacteria (X 10^6 CFU/gm)</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>Fungi (X 10^6 CFU/gm)</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Actinomycetes (X 10^6 CFU/gm)</td>
<td>30</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

We wish to thank the Management, Secretary and the Principal of Sri Ramakalyani college for providing all facilities to carry out this study.

REFERENCES:


Evaluation of Phage Therapy to Treat Experimental Infections in Mice

R Gowri Sankar*, V Madhusudhan and R Palaniappan
Post Graduate Department of Microbiology, Sri Paramakalyani College, Alwarkurichi - 627 412, India.

The use of bacteriophage to treat bacterial infection is a novel approach and is being seriously considered. Two common bacterial pathogens namely, Pseudomonas aeruginosa and Streptococcus pyogenes were isolated from a rural hospital. Bacteriophages specific for these pathogens were isolated from a domestic sewage. Test animals (mice) were injected with the bacterial pathogen through the intra-peritoneal route. Their phage partners were also administered subsequently through the same route. Animals protected with phage ingestion exhibited no sign of illness when compared to the unprotected ones. This protection could be attributed to the marked reduction of the pathogen load in the tissues of the animal in response to the presence and persistence of phage.

Key words: Bacteriophage, Pseudomonas aeruginosa, Streptococcus pyogenes, phage therapy.

Nosocomial infections represent a major hazard in health care facilities. Despite the many advances in modern medicine, these hospital-borne infections still pose a significant risk to patients (1). A remarkable proportion of this is caused by bacteria (88%). Most of these can be attributed to Pseudomonas aeruginosa followed by Escherichia coli and Streptococcus pyogenes. In the treatment of nosocomial infections, the choice of antibiotics has been challenged due to the abrupt emergence of antibiotic resistant strains (2,3). There had been claims of the successful use of bacteriophage in the treatment of bacterial infections (4). However, considerable debate on the use of bacteriophage as therapeutic agent is still on, due to its intermittent failures. It is generally thought that phages are of little value in controlling bacterial infections in man. The main reasons for their apparent failures are thought to be, low activity in vivo, presence of phage antibodies in the blood of the infected animal and rapid emergence of phage resistant mutants among pathogens during treatment (5). In this background, the present study was proposed to investigate the validity of phage therapy and changes in the microbial population during the course of the therapy.

Materials and Methods

Isolation of pathogens: Bacterial pathogens for this study were isolated from a rural hospital. Samples were collected through dual swab technique (wet and dry swab) at various areas in the hospital such as labour theatre, operation theatre, wards, etc. The swab samples were transported in sterile 1.0% peptone water kept in an ice box. Nutrient agar (Hi-Media, Bombay) was used to enumerate total bacterial load (TBL). From the TBL plate morphologically dissimilar colonies were pure cultured and were characterised using standard techniques (6).

Isolation of phages: Bacteriophages were isolated from sewage samples obtained from a nearby students hostel by the method of Smith and Huggins (7). Sewage water (50 ml) was collected in a sterile conical flask and treated with a few drops of chloroform. To this, equal volume of sterile nutrient broth and 1.0 ml of the 24 h old broth culture of P. aeruginosa or S. pyogenes was added. The samples inoculated with bacterial pathogens were incubated at 37°C for 12-24 h in a water bath shaker. After 12-24 h, the lysate was shaken with few drops of chloroform for about 10 min, centrifuged at 10,000 rpm for 10 min and the supernatant (lysate) was stored over a few drops of chloroform and this was used as phage stock. Phage plaques appeared in double layer plating with P. aeruginosa and S. pyogenes, (specific to these host pathogens) were individually taken, retested by plating from single plaque and finally taken as single phage specific for P. aeruginosa and S. pyogenes.

Experimental infections in mice: Germ free, male albino mice of the same race with an average weight of 180 ± 20 g were used in this study. They were caged singly and were fed with fresh greens and vegetables during the experimental period. Mice (6 in each group) were inoculated with 0.1 ml of bacterial suspensions of P. aeruginosa (1.75 x 10^4 cells g^-1 body wt of mouse) and S. pyogenes (4.35 x 10^4 cells g^-1 body wt of mouse ) intra-peritoneally. After a time lap of 6 h, corresponding bacteriophage lysate, at varied dose of (0.5 ml and 1.0 ml) was also administered intra-peritoneally. The inoculum concentration of phage with P. aeruginosa as the host was 5.5 x 10^4 PFU g^-1 body wt and 2.8 x 10^4 PFU g^-1 body wt at 1.0 ml and 0.5 ml dose respectively.

*Corresponding author
The inoculum concentration of phage with \textit{S. pyogenes} as the host was 4.0 x 10^6 PFU g⁻¹ body wt and 2.0 x 10^6 PFU g⁻¹ body wt at 1.0 ml and 0.5 ml dose respectively.

\textbf{Bacterial and phage counts in tissues:} After the manifestation of terminal illness, such as lethargy, weak response to stimuli, loss of appetite, breathlessness, etc, the animal was sacrificed and the concentration of \textit{P. aeruginosa} and \textit{S. pyogenes} and phage load in the animal was enumerated. At the end of the experimental period, even those animals which did not exhibit symptoms of illness were sacrificed and analysed for experimental purposes. The entire viscera of the test animal was macerated in a surface sterilised mortar and pestle, serially diluted with sterile 1.0 % peptone water and plated for bacterial pathogen and phage titre. \textit{Pseudomonas} isolation agar and azide blood agar were used to enumerate \textit{P. aeruginosa} and \textit{S. pyogenes} respectively. Phages were enumerated on nutrient agar using double layer technique with \textit{P. aeruginosa} and \textit{S. pyogenes} as the host.

\textbf{Results and Discussion}

The bacterial strains were isolated from a rural hospital and were designated as \textit{P. aeruginosa} SPK01 and \textit{S. pyogenes} SPK02. The phage isolated with \textit{P. aeruginosa} SPK01 as the host was coded as phage PA01 and the phage isolated with \textit{S. pyogenes} as the host as phage SP02.

No mortality was observed in the uninfected and phage control animals and they were sacrificed only on academic interest. On the contrary, mice inoculated with bacterial pathogens alone were the first to express symptoms of terminal illness and had a very short survival period of 36-40 ± 8 h (Table 1). On the other hand, mice treated with double dose of phage lysate PA01-5.5 x 10^6 ; SP02- 4.0 x 10^6 PFU g⁻¹ body wt), exhibited no sign of illness and was comparable with that of control mice. In the case of mice treated with lower concentration of lysate (PA01 - 2.8 x 10^4 ; SP02- 2.0 x 10^4 PFU g⁻¹ body wt) the expression of symptoms were weak which includes, lethargy, reduced appetite but had covered completely within 24-36 h. Soothil (4) ported that the phage dose of 1.8 x 10^7 PFU ml⁻¹ infected the infected mice significantly over the control.

Further analysis of the animal tissues indicates a marked reduction in the bacterial load in response to phage administration (Table 2). This is suggestive of possible lytic reduction of the pathogen load by the phage \textit{in vivo} in the test animal. Th bactericidal effect of bacteriophage on their respective hosts \textit{in vivo} could explain the absence or remission of morbidity and survival of the test animal administered with both bacterial pathogen and phage. Similar observations were reported with experimental \textit{E. coli} infections in mice by Smith and Huggins (5).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Mice injected with} & \textbf{Inoculum dose (g⁻¹ body wt)} & \textbf{Animals survived (#)} \\
\hline
\hline
Control & - & 6 \\
\hline
\textit{P. aeruginosa} SPK01 & 1.75 x 10^4 & 6* \\
\textit{P. aeruginosa} SPK01 + Phage PA01 & 1.75 x 10^4  \text{ 2.80 x 10^4} & 6 \\
\textit{P. aeruginosa} SPK01 + Phage PA01 & 1.75 x 10^4  \text{ 5.50 x 10^4} & 6** \\
\textit{S. pyogenes} SPK02 & 4.35 x 10^4 & 6 \\
\textit{S. pyogenes} SPK02 + Phage SP02 & 4.35 x 10^4  \text{ 2.00 x 10^4} & 6 \\
\textit{S. pyogenes} SPK02 + Phage SP02 & 4.35 x 10^4  \text{ 4.00 x 10^4} & 6 \\
Phage PA01 & 5.50 x 10^4 & 6 \\
Phage SP02 & 4.35 x 10^4 & 6  \\
\hline
\end{tabular}
\caption{Survival of mice after 144 h of infecting with \textit{P. aeruginosa} SPK01 and \textit{S. pyogenes} SPK02 and Phage treatment.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Mice injection With} & \textbf{Bacterial count (CFU g⁻¹ body wt)} & \textbf{Phage dose} & \\
\hline
\hline
\textit{P. aeruginosa} SPK01 & 6.3 x 10^6 & 3.80 x 10^6 & \\
\textit{P. aeruginosa} SPK01 + Phage PA01 & 2.0 x 10^6 & 3.80 x 10^6 & \\
\textit{P. aeruginosa} SPK01 + Phage PA01 & 4.0 x 10^6 & 3.80 x 10^6 & \\
\textit{S. pyogenes} SPK02 & 8.3 x 10^8 & 3.80 x 10^6 & \\
\textit{S. pyogenes} SPK02 + Phage SP02 & 2.9 x 10^5 & 3.80 x 10^6 & \\
\hline
\end{tabular}
\caption{Population of \textit{P. aeruginosa} SPK01 and \textit{S. pyogenes} SPK02 in the viscera of mice.}
\end{table}

From our experimental data, ingestion dose of the phage seems to have a significant impact on the morbidity in the test animal as the animal protected with lower dose of phage had initially exhibited mild symptoms of illness such as lethargy and reduced food intake and later intake. Hence, this study emphasises the need for proper optimisation of phage dose for effective control of bacterial infection without the risk of onset of the disease.
This study clearly demonstrates the relevance of phage therapy, as animals administered with the phage particles survived the bacterial infection with weak or no sign of illness. This protection could be attributed to the lytic interaction of phage with pathogen in the animal as the PFU of phages increased in the mice infected with the specific host pathogen (Table 3).

Table 3. Phage PA01 & SP02 load in the viscera of mice.

<table>
<thead>
<tr>
<th>Mice injection With Phage count (PFU g body wt)</th>
<th>PA01</th>
<th>SP02</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa + Phage PA01 (Phage dose 2.8 x 10⁶)</td>
<td>1.0 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa + Phage PA01 (Phage dose 5.5 x 10⁴)</td>
<td>2.0 x 10⁵</td>
<td></td>
</tr>
<tr>
<td>S. pyogenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pyogenes + Phage SP02 (Phage dose 2.0 x 10⁴)</td>
<td></td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td>S. pyogenes + Phage SP02 (Phage dose 4.0 x 10⁴)</td>
<td></td>
<td>4.0 x 10⁴</td>
</tr>
<tr>
<td>Phage PA01</td>
<td>6.0 x 10³</td>
<td></td>
</tr>
<tr>
<td>Phage SP02</td>
<td></td>
<td>4.0 x 10³</td>
</tr>
</tbody>
</table>

Further assessment of the specificity and safety profile of the phage particle in the hospital environment would be useful in employing the bacteriophage in place of disinfectants, germicides and as a bio-therapeutic agents.

Acknowledgements

The authors are grateful to the Secretary, Principal and the management of Sri Paramakalyani College, Alwarkurich for providing the facilities to carry out this study.

References

1. Istre GR, J Infect Dis, 156 (1987) 732.