Occurrence of chromium resistant thermotolerant coliforms in tannery effluent

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Twenty six thermotolerant strains resistant to high levels of chromium (50-250 μg/ml) were isolated from treated tannery effluent. They were also found resistant to multiple heavy metals and antibiotics. Majority of them were resistant to copper and bacitracin. Nine strains representing different resistance patterns were selected for plasmid profile and conjugation studies. Agarose gel electrophoresis results revealed that 6 strains harboured a single plasmid, whereas 3 strains exhibited 2 plasmid bands. Among antimicrobials, co-trimazole and bacitracin and among metals, Cu²⁺, Cd²⁺, Zn²⁺ and Ni²⁺ resistance were transferred most frequently at variable rates. However, chromium resistance was transferred in 6 strains with a frequency ranging 19-49 x 10⁻². Resistance to Co²⁺ and Hg²⁺ did not transfer under environmental conditions. Among the nine strains, three were found predominantly uropathogenic Escherichia coli (UPEC) serotype 04, whereas two strains were untypable. In addition, 4 transconjugants also showed a positive result after serotyping.

Keywords: Antibiotic resistance, Conjugation, Escherichia coli, Heavy metals, Plasmid, Tannery effluent, Thermotolerant E. coli.

Chromates and dichromates constitute a significant percentage of discharge from tanneries and other industries that pose a serious problem for environmental quality. Elevated concentrations of metals in the environment have been observed to cause a wide range of impacts on microbes, plants, animals and human population as well. The presence of chromium in the environment exerts an inhibitory action on most microorganisms, but also promotes the selection of resistant species. The metal resistance is often associated with resistance to single or multiple antibiotics, which may be a desirable characteristic for an organism in a natural environment of mixed population. Among natural communities, the development of metal and antibiotic resistance could be greatly enhanced by the horizontal transfer of genetic information and for the establishment of new genetic traits in the diverse environment.

Since the treated tannery effluent is finally discharged in both urban and rural environments and eventually enters the food chain, adequate attention should be paid towards the evaluation of bacteriological quality of tannery effluent prior to discharge into the environment. However, information is not available on incidence of thermotolerant coliforms in tannery effluent. Some thermotolerant coliforms are well recognized as fecal contaminants in water bodies. Quantification of these coliforms is an integral part of quality assessment of water. Method used to distinguish fecal coliforms from total coliforms is elevated temperature test proposed by Eijkman. Most coliforms grow at 35°C with ability to ferment lactose, with the product ion of acid and gas, but only fecal coliforms grow at about 45°C. The strains may be pathogenic or non-pathogenic depending upon their antigens. Serotyping is one method for characterization of clinical isolates. These bacteria if survive and propagate in the tannery effluent may have an effect on public health. Apart from R-factors, bacteria may also harbour plasmids that transmit enteropathogenicity. Large amounts of such contaminated tannery effluents reach eventually to the ground water sources and further aggravate the situation. Thus, the environmental spread of metal resistant bacteria provides a useful indication of the prevailing conditions.

This study was aimed to isolate thermotolerant coliforms from treated tannery effluent, to ascertain their resistance to chromium and other heavy metals/antibiotics, degree of pathogenesis and ability to transfer the chromium resistance in association with pathogenesis.

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Materials and Methods

Sampling and isolation of thermotolerant coliforms—The tannery effluent samples were collected in sterile glass bottles from a common effluent treatment plant (CETP), Unnao (India), transported in an ice box to the laboratory and processed within 6-8 hr of collection. The effluent samples were inoculated into brilliant green bile broth (BGBB) and peptone water (Hi-Media, India) supplemented with 50-250 µg/ml of Cr(6+) as K2Cr2O7 (Qualigens, India) and were incubated at 45°C. Gas production in BGBB and indole formation in peptone water after 48 hr of incubation confirmed the presence of coliforms. Further, suitably diluted samples from positive BGBB test were plated on Mac-Conkey agar (MCA) (Hi-Media, India) plates supplemented with different concentrations of Cr(6+) (50 to 250 µg/ml). Randomly selected colonies were purified by repeated streaking on agar plates.

Identification and serotyping of isolates—The scheme of Cowan and Steel was followed for identification of the isolates. The biochemical tests performed were oxidase, urease, carbohydrate catabolism, starch, gelatin and casein hydrolysis, H2S production, indole production, methyl red, Voges-Proskauer and citrate utilization. The results were interpreted according to Bergey's Manual of Determinative Bacteriology. The identification of the strains was confirmed from Institute of Microbial Technology, Chandigarh, India. These isolates were serotyped at the National Salmonella and Escherichia Research Centre, Central Research Institute, Kasauli, India.

Minimal inhibitory concentration (MIC)—MIC of chromium or other metals [MnCl2, As2O3 (E. Merck), CuSO4,5H2O, ZnSO4,7H2O, Co(NO3)2,6H2O, CdCl2 and HgCl2 (Qualigens)] was determined by the agar dilution method. The identification of the strains was confirmed from Institute of Microbial Technology, Chandigarh, India. These isolates were serotyped at the National Salmonella and Escherichia Research Centre, Central Research Institute, Kasauli, India.

Antibiotic susceptibility test—Antibiotic sensitivity was determined on Muller-Hinton medium (Hi-Media, India) by the disc diffusion method. The antibiotic discs were placed on freshly prepared lawns of each strain on agar plates, incubated at 35°-37°C for 24 hr and examined for the inhibition zones. Discs containing the following antibiotics (µg per disc) were used: gentamycin (10), polymixin-B (50), chloramphenicol (30), kanamycin (30), tetracycline (30), bacitracin (10), streptomycin (25), ampicillin (25), carbencillin (10), co-trimazole (25), nalidixic acid (30) and cephaloridine (30).

Conjugal R-transfer—Nine strains, representing different resistance patterns and higher MIC values, were tested for their ability to transfer their resistance to recipient E. coli K12 J62 strain (lac+, pro+, his+, trp+, nal+) (E. coli K12 J62 strain). Overnight grown cultures (0.1 ml) were inoculated into 10 ml peptone water (Hi-Media), incubated for 6 hr and then 0.1 ml of each donor and recipient cultures were mixed and incubated for 18 hr at 30°C for conjugation. Transconjugates were selected on MCA plates containing appropriate antibiotics/heavy metals. The rates of plasmid transfer were expressed as the number of transconjugants formed per donor.

Plasmid curing—Tubes containing 10 ml of peptone water was supplemented with acridine orange (20 µg/ml), inoculated with 0.1 ml overnight culture and incubated at 37°C for 24 hr. Appropriate dilutions of the culture were plated on MCA plates and incubated for 24 hr. Resulting colonies were tested for loss of plasmid on MCA plates incorporated with the appropriate antibiotic/metal ion.

Plasmid isolation and gel electrophoresis—The alkali lysis method was used to isolate plasmids from the nine E. coli strains. These plasmids were resolved by agarose gel electrophoresis according to the standard procedure.

Results

Isolation, identification and MIC determination—The bacterial counts (CFU/ml) in positive BGBB test (for thermotolerant coliforms) was measured on chromate supplemented plates (Table 1). The results revealed the presence of total thermotolerant coliforms in the range of 4×10^2-5.25×10^3 cfu/ml. The bacterial count on plates for 24 hr and examined for the inhibition zones. Discs containing the following antibiotics (µg per disc) were used: gentamycin (10), polymixin-B (50), chloramphenicol (30), kanamycin (30), tetracycline (30), bacitracin (10), streptomycin (25), ampicillin (25), carbencillin (10), co-trimazole (25), nalidixic acid (30) and cephaloridine (30).

<table>
<thead>
<tr>
<th>Conc. of Cr(6+) (µg/ml)</th>
<th>10^3 cfu/ml</th>
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</thead>
<tbody>
<tr>
<td>50</td>
<td>525</td>
</tr>
<tr>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>200</td>
<td>17</td>
</tr>
<tr>
<td>250</td>
<td>4</td>
</tr>
</tbody>
</table>
decreased with increasing concentration of Cr\(^{3+}\) indicating their sensitivity to higher concentration of chromate.

A total of twenty-six chromium resistant thermotolerant coliforms were isolated and identified as *Escherichia coli*. Majority of the isolates (38%) were tolerant to 101-150 µg/ml of chromate, while 27% of the isolates were resistant to 151-200 µg/ml of chromate. Approximately 19% isolates were resistant to chromate concentration 201-250 µg/ml, while 15% isolates had their MIC ranging between 50-100 µg/ml.

**Metal and antibiotic resistance**—The chrome resistant thermotolerant *E. coli* were also found resistant to multiple metals. The isolates were resistant to different heavy metals at 200 µg/ml, except for Co\(^{2+}\) (175 µg/ml) and Hg\(^{2+}\) (25 µg/ml). The decreasing order of metal resistance in *E. coli* was—Cu\(^{2+}\) (80.8%), As\(^{3+}\) (77.0%), Cd\(^{2+}\) (77.0%), Ni\(^{2+}\) (69.0%), Zn\(^{2+}\) (69.0%), Co\(^{2+}\) (61.0%), Hg\(^{2+}\) (50%) and Mn\(^{2+}\) (19.2%). Regarding antibiotic sensitivity, all twenty six strains were resistant to bacitracin, but susceptible to gentamycin. Moderate resistance was observed for polymixin-B (48.5%), co-trimazole (39.1%), cephaloridine (27.1%), streptomycin (20.4%), ampicillin (16.7%) and tetracycline (13.5%). Low level of resistance was observed in chloramphenicol, carbencillin, nalidixic acid and kanamycin (8.3-3.8%).

Significant number (73.1%) of *E. coli* resistant to Cd\(^{2+}\) and As\(^{3+}\) were also resistant to bacitracin. However, all tetracycline and kanamycin resistant isolates were also resistant to polymixin-B. Around 15% isolates resistant to co-trimazole were also resistant to bacitracin. Furthermore, 11.5% were found resistant to multiple metals (Zn\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) and As\(^{3+}\)) and antibiotics (polymixin-B and bacitracin). About 11.5% strains tolerant to combinations of Cu\(^{2+}\), Cd\(^{2+}\) and As\(^{3+}\) were resistant to co-trimazole.

**Virulence factor of thermotolerant *E. coli***—Nine *E. coli* strains and their transconjugants were serotyped to assess their degree of pathogenicity (Table 2). Three of them were found predominantly uropathogenic *E. coli* (UPEC) serotype 04. Four strains, each was of enterotoxigenic *E. coli* (ETEC) 025 and ETEC 0168, shiga toxin producing *E. coli* (STEC) 0157 and STEC 0103, two strains were found untypable.

**Plasmid transfer to *E. coli* K12 J62**—Among the nine strains, seven were able to transfer different heavy metals and antibiotics resistance to *E. coli* K12 J62 (Table 2). While six of them were able to transfer chromium resistance at a frequency of 19-49x10\(^{-2}\). Traits that transferred most frequently were co-trimazole and bacitracin and among metals traits were Cu\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\) and Ni\(^{2+}\).

Among metals, arsenic resistance was transferred relatively at low frequency; cobalt and mercury resistance were not transferred under experimental conditions. The frequency of transfer of Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\) and Ni\(^{2+}\) resistance ranged from 8-38×10\(^{-2}\).

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Resistance pattern</th>
<th>Curing pattern</th>
<th>Trait transferred</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zn(^{2+}), Ni(^{2+}), Cu(^{2+}), Cd(^{2+}), Cr(^{6+}), Cu(^{2+}), Co(^{2+}), Cd(^{2+}), Cd(^{3+})</td>
<td>Cu(^{2+}), Cr(^{6+}), Co(^{2+}), Cd(^{3+})</td>
<td>Ni(^{2+}), Cu(^{2+}), Cd(^{3+}), Cr(^{6+}), B, CO</td>
<td>ETEC-025</td>
</tr>
<tr>
<td>2</td>
<td>Zn(^{2+}), Cu(^{2+}), As(^{3+}), Hg(^{2+}), B, S</td>
<td>Cu(^{2+}), B</td>
<td>No conjugation</td>
<td>UPEC-04</td>
</tr>
<tr>
<td>3</td>
<td>Cu(^{2+}), Cr(^{6+}), Cd(^{2+}), As(^{3+}), Hg(^{2+}), B, CR</td>
<td>Cu(^{2+}), Cr(^{6+}), Hg(^{2+}), CR, B,</td>
<td>Cr(^{2+}), B, CO, As(^{3+}), CR</td>
<td>UPEC-04</td>
</tr>
<tr>
<td>4</td>
<td>Cu(^{2+}), Cr(^{6+}), Cd(^{2+}), As(^{3+}), B, CO</td>
<td>Cr(^{2+}), Cd(^{3+}), B</td>
<td>Cu(^{2+}), Cr(^{6+}), Cd(^{3+}), B, CO</td>
<td>ETEC-0168</td>
</tr>
<tr>
<td>5</td>
<td>Cu(^{2+}), Hg(^{2+}), S, CR, CN, CO, A, T, K, C, B, PB</td>
<td>Cr(^{2+}), Hg(^{2+}), B</td>
<td>Cr(^{2+}), Cr(^{6+}), A, CO, PB</td>
<td>STEC-0157</td>
</tr>
<tr>
<td>6</td>
<td>Zn(^{2+}), Cu(^{2+}), Cr(^{6+}), Cd(^{2+}), Ni(^{2+}), B</td>
<td>Zn(^{2+}), Cu(^{2+}), Cr(^{6+}), Cd(^{2+}), As(^{3+}), B</td>
<td>Zn(^{2+}), Cu(^{2+}), Cr(^{6+}), Cd(^{3+}), As(^{3+}), CR</td>
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<tr>
<td>7</td>
<td>Zn(^{2+}), Ni(^{2+}), Cu(^{2+}), Cd(^{2+}), Hg(^{2+}), B</td>
<td>Zn(^{2+}), Ni(^{2+}), Cu(^{2+}), Cd(^{3+}), As(^{3+}), B, Hg(^{2+}), B</td>
<td>Zn(^{2+}), Cu(^{2+}), Cr(^{6+}), Cd(^{3+}), As(^{3+}), CR</td>
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</tr>
<tr>
<td>8</td>
<td>Zn(^{2+}), Cu(^{2+}), Cd(^{2+}), As(^{3+}), Ni(^{2+}), B</td>
<td>Zn(^{2+}), Cu(^{2+}), Cd(^{3+}), Ni(^{2+}), B</td>
<td>Zn(^{2+}), Cu(^{2+}), Cd(^{3+}), Ni(^{2+}), B</td>
<td>Untypable</td>
</tr>
<tr>
<td>9</td>
<td>Cu(^{2+}), Co(^{2+}), Cd(^{2+}), Hg(^{2+}), B, CO</td>
<td>Co(^{2+}), Cd(^{3+}), B</td>
<td>Cu(^{2+}), Cd(^{2+}), Co(^{2+}), B, CO</td>
<td>UPEC-04</td>
</tr>
</tbody>
</table>

As\(^{3+}\) = arsenic; Cd\(^{2+}\) = cadmium; Co\(^{2+}\) = cobalt; Cu\(^{2+}\) = copper; Hg\(^{2+}\) = mercury; Mn\(^{2+}\) = manganese; Ni\(^{2+}\) = nickel; Zn\(^{2+}\) = zinc; A = ampicillin; B = bacitracin; C = chloramphenicol; CN = carbencillin; CO = co-trimazole; CR = cephaloridine; K = kanamycin; PB = polymixin-B; S = streptomycin; T = tetracycline.
Among antibiotics, co-trimazole and bacitracin resistance was transferred at a frequency ranging from 21-38 x 10⁻² and 11-38 x 10⁻², respectively. The average transfer rates for cephaloridine, ampicillin and polymixin-B were 2 x 10⁻¹, 2.7 x 10⁻¹ and 2.6 x 10⁻¹, respectively. Additionally, pathogenicity might have also transferred in four strains (no.- 1, 3, 4 and 6) as their transconjugants showed the respective pathogenic types after serotyping.

Plasmid characteristic—Nine strains of E. coli representing different resistance patterns harboured one or more plasmids (not shown). Of the nine, six strains harboured only a single plasmid, whereas, three strains showed the presence of two plasmid bands. Resistance to chromium and bacitracin was completely eliminated by acridine orange in all nine strains (Table 2). Curing of transferable markers revealed elimination of resistance to Cr₆⁺, Zn²⁺, Ni²⁺, bacitracin and cephaloridine in all the strains, whereas, Cd²⁺ and Cu²⁺ resistance was lost in three (nos.-1, 4 and 6) and four (nos.-1, 3, 6 and 8) strains, respectively. Resistance to As³⁺, co-trimazole, polymixin-B and ampicillin was transferred during conjugation but was not cured by acridine orange.

Discussion

The present study highlighted the prevalence of chromate resistant thermotolerant coliforms in the treated tannery effluent indicating fecal contamination and inadequate treatment. Therefore, information on types, numbers and characteristics of bacteria are important in understanding the quality of treated effluent as it is released in the environment for various purposes. Chromium resistant bacteria isolated from tanneries have been studied by many researchers, but to our knowledge there is no report available on the detection of thermotolerant coliforms in tannery effluent. Detection of coliforms especially thermotolerant types indicates fecal pollution of water. Hence, this study suggests the need for regular biological monitoring of the treated effluent. This study assumes even greater significance from the public health point of view because some of the thermotolerant E. coli were found pathogenic, based on their serotypes.

Enterobacteria are known to cause a number of gastrointestinal disorders in man and animals. In humans, ETEC is a major cause of diarrhoea in young children from developing countries and adults from industrialized countries travelling to these regions. Fecal contamination of food and drinking water is the major route of infections for humans. UPEC gets adhered to the epithelial cells of the urinary tract and represent by far the most frequent cause of urinary tract infections in humans. STEC is associated with infant diarrhoea and hemorrhagic colitis in human. In addition to these typed E. coli, there are other types of E. coli, which harbour certain virulence genes whose combination does not give them a particular pathogenic phenotype. However, presence of non-pathogenic E. coli in water is a clear sign of bad hygiene. Further, such strains, which are mainly not typed, might constitute a huge environmental genetic reservoir for virulence and might be leading factors in creation of new pathogens. The genome of E. coli is of high plasticity allowing it to gain or lose genes at a relatively high frequency. Since many virulence genes in E. coli are located on plasmids, strains with new combinations of virulence genes might emerge in future.

Natural habitats are generally characterized by co-existence of a large number of toxic and non-toxic cations and therefore, it is necessary to study multiple metal effects. The present study indicated that in addition to chromium resistance, the bacteria were also found tolerant to multiple metals with differences in their level of resistance. In addition to metal tolerance, these microbes also exhibited resistance to different antimicrobials. Microorganisms resistant to antibiotics and tolerant to metals appeared as the result of exposure to metal contaminated environments, which caused coincidental co-selection of resistance factors for antibiotics and heavy metals. Association between resistance to antibiotics and heavy metals has been reported by several researchers. In all these cases, genes encoding resistance to metals and antibiotics were located on plasmids. Our results indicated that, in majority of isolates both metal/antibiotic resistance and virulence were plasmid mediated. Under environmental conditions of metal stress, such population will adapt faster by the spread of R-factors, thereby causing serious constraints to therapeutical measures. Also R-plasmids can severely impede the quick and easy treatment of bacterial infections.

Thus, we conclude that tannery effluent provides enriched medium to propagate and spread thermotolerant E. coli. In this study, we have isolated thermotolerant E. coli from tannery effluent. The possession of antibiotic resistance and pathogenic
character of these bacteria will lead to a serious public health hazard. Hence, there is a necessity to limit the dissemination of R-bacteria in polluted water bodies. For this, effort should be made to develop adequate technology for effluent treatment. As the treated tannery effluent is finally released into the environment for irrigation and other purposes, the microbes present in the effluents and wastes may cause many newly emerging diseases.

References