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Original Article

Tellurite reduction potential of bacteria isolated from industrial wastewater

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Abstract

The aim of the present study was to isolate tellurite resistant bacteria from industrial wastewater. Two bacterial isolates were identified as *Staphylococcus epidermidis* and *Staphylococcus lactis* on the basis of biochemical tests and 16S rRNA sequence. The minimum inhibitory concentration of both isolates showed fair growth up to 1 mM of $K_2Te_2O_3$. Both strains showed maximum growth at 37ºC and pH of 7. The bacterial growth in the presence of metal ions was delayed as compared to the control. High tellurite reduction potential was shown 98% (1f) and 97% (2b) after 24 h of incubation. Glucose 6 phosphate dehydrogenase (G6PDH) activity was increased to 279% (1f) and 150% (2b) when compared to the respective control indicating the ability to tolerate tellurite stress. Differential pattern of bacterial proteins in tellurite stress was obtained by SDS-PAGE. Both bacterial strains can be utilized to convert toxic tellurite into its elemental form from the contaminated sites.

Keywords: Tellurite; *S. epidermidis*; *S. lactis*; glucose 6 phosphate dehydrogenase; tellurite reduction

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INTRODUCTION

Tellurium (Te) is a trace element that is essential in small amount, but it is toxic at very high concentration. It was first discovered by Muller (Dittmer, 2003) in 1782 and tellurium came from the Latin word “tellus”, which means “Earth” (Weeks, 1956). Te is the ninth rare metal on Earth’s crust and is widely distributed in nature. It is very toxic at very low concentration even at < 1 µg/ml (4 µM) (Summers and Jacoby, 1977; Summers and Silver, 1978). Tellurium is used for many purposes such as vulcanization of rubber to increase their resistance to heat, aging as well as abrasion. It is used to make alloys of copper and other metals such as steel, lead, and bronze in which it used to make resistant to corrosion as well as used in manufacturing of jewelry. Te has some medical importance; it is used for the treatment of some diseases such as dermatitis, eye infections, tuberculosis, leprosy and syphilis caused by bacteria (Cooper, 1971; Ba *et al*., 2010). Tellurium is a very toxic metal for many microorganisms and it is used for several industries including electronics, chemical and metallurgy, as well as for agricultural purposes, which are responsible for its high toxic levels in the environment and presence of toxic tellurium oxyanion effluents in soil and water (Klevay, 1976; Taylor, 1996). Black color precipitates are produced by many microorganisms is due to presence of potassium tellurite, known as metallic tellurium (King and Davis, 1914; Morton and Anderson, 1941). Tellurium oxyanions (TeO_3^{2-}), is extremely toxic for many microorganisms at very low concentration 1µg/ml including *Escherichia coli* (Taylor, 1999).Many bacteria have ability to survive at high concentration of tellurium metal and are able to convert highly toxic form of tellurium to less toxic form such as tellurite to elemental tellurium(Sabaty *et al*., 2001; Ollivier *et al*., 2008). Elemental tellurium present in insoluble form and it forms black color around some bacterial species (Chasteen and Bentley, 2003; Amoozegar *et al*., 2008; Chasteen *et al*., 2009).

MATERIALS AND METHODS

Isolation of tellurite resistant bacteria

Wastewater samples were collected from industrial area of Sheikhupura, a city near Lahore. The tellurite resistant bacteria were isolated by plating the wastewater samples on L-

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agar plates. LB agar was prepared by dissolving yeast extract (5g), trypton (10g), NaCl (5g) and agar (15g) and the volume made up to 1000 mL by adding distilled water. All the ingredients were properly mixed and then autoclaved for 15 min. For the selection of tellurite resistant bacteria, the medium was supplemented with 0.5mM concentration of $K_2Te_2O_3$. Dilutions were made of these samples up to 10^{-4} in autoclaved distilled water and these dilutions were then spread on L-agar plates. L-agar prepared by using recipe from manual (Cappuccino and Sherman, 2002).By dissolving yaest extract (5g), trypton (10g), NaCl (5g) and agar (15g) and the volume made up to 1000 mL by adding distilled water.

Determination of minimum inhibitory concentration (MIC)

After plating and colony morphology, minimum inhibitory concentrations (MIC) of tellurite resistant bacteria were checked. The medium was supplemented with 0.05, 0.1, 0.5 and l mM concentration of $K_2Te_2O_3$. For this experiment, minimal salt (M9) broth ($FeSO_4.7H_2O$ 0.015g, KH_2PO_4 4.7g, $MgSO_4.7H_20$ 1g, $CaCl_2.2H_2O$ 0.01g, Na_2HPO_4 0.12g, NH_4NO_3 4g, $MnSO_4.4H_2O$ 0.01g, pH 7-7.2) was prepared. Different heavy metals such as Cr^{+6} (K_2CrO_4), Cu^{+2} ($CuSO_4$), Cd^{+2} ($CdCl_2$), Zn^{2+} ($ZnCl_2$), Pb^{2+} ($PbCl_2$), Ni^{2+} ($NiCl_2$) and arsenic (sodium arsenite) were supplemented in broth at different concentrations. Each isolate was inoculated in broth of different metals separately and incubated for 24 h at 37°C.

Identification of bacterial isolates

Morphological as well as biochemical characteristics were determined. Gram reaction, catalase test, mannitol fermentation test, pigmentation test and shape of colony, color of colony, margin, texture and light transparency were also checked (Cappuccino and Sherman, 2002). The genomic DNA from the selected bacteria was isolated according to Masneuf-Pomarade *et al*. (2007) and 16S rRNA gene amplification was done by universal primers (RS-1 and RS-3). The conditions followed for amplification were as follows: Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. The PCR products were subjected to 1% agarose gel electrophoresis for analysis in 1X TAE buffer with ethidium bromide and visualized under UV transilluminator. PCR products were purified through Fermentas Gene Jet Gel Extraction kit (#K0691) and were sent to CEMB, Thokar Niaz Baig, Lahore for sequencing.

Heavy metal resistance

For this experiment, minimal salt (M9) broth ($FeSO_4.7H_2O$ 0.015g, KH_2PO_4 4.7g, $MgSO_4.7H_20$ 1g, $CaCl_2.2H_2O$ 0.01g, Na_2HPO_4 0.12g, NH_4NO_3 4g, $MnSO_4.4H_2O$ 0.01g, pH 7-7.2) was prepared. Different heavy metals such as Cr^{+6} (K_2CrO_4), Cu^{+2} ($CuSO_4$), Cd^{+2} ($CdCl_2$), Zn^{2+} ($ZnCl_2$), Pb^{2+} ($PbCl_2$), Ni^{2+} ($NiCl_2$) and arsenic (sodium arsenite) were supplemented with 0.1 mM each metal separately at 37°C for 24 h. The process was repeated with high concentrations of each metal until the growth of the isolate was inhibited. The minimum metal concentration at which bacterial isolate did not show growth was considered as its MIC.

Bacterial growth characteristics

Physiological characteristics of both bacterial isolates were also determined. Both bacterial isolates were incubated at different temperature 25-45°C and pH 5-9. The pH of L-broth was adjusted by using HCl or NaOH. Each isolate was inoculated in broth of different pH separately and incubated for 24h at 37ºC.Bacterial growth curves were determined with respect to time in M9 which was supplemented with 0.5mM concentration of $K_2Te_2O_3$. Two sets for each isolates were prepared, and replica for each set also prepared. One set with metal and one set without metal were prepared and all flasks were placed in shaking incubator. The growth was measured in terms of optical densities at 600 nm.

Tellurite reduction potential of bacterial isolates

For measuring the tellurite reduction potential of both isolates, M9 medium was prepared. A loopful of bacterial culture was taken from 24 h freshly prepared overnight culture in two flasks separately for each isolate. Two flasks without bacterial culture were prepared which acted as control. After inoculum, the flasks were incubated 37ºC for overnight, 4ml of medium was taken from each flask in falcon tube and then centrifuged at 6000 rpm for 10 min until supernatant clear. To supernatant, $NaBH_4$ (3.5mM), fresh stock solution of $NaBH_4$ were prepared after every time interval, was added. After addition of $NaBH_4$ solution,

bubbling was produced. To remove bubbling, the falcon tubes were vortexed. Tellurite amount was estimated in the supernatant and pellet appeared black in color due to presence of tellurium metal. Optical density was taken after 0, 4, 8, 12 and 24 h at 500nm.

Glucose 6 phosphate dehydrogenase assay

LB broth was prepared, autoclaved and each isolate was inoculated in broth and incubated for 24 h at 37^{o}C. Centrifugation was done at 14000 rpm for 10 min. Discard the supernatant, take the pellet and lysate pellet with 50µl potassium phosphate buffer, 0.5µl EDTA and 950µL of water. Then, 250µL lysate was taken and 0.5ml of 10% TCA was added and centrifuged for 5 min. The pellet was discarded and supernatant was taken into another eppendorf. Crude enzyme was prepared and was used for enzyme assay. For enzyme activity, add 250µL potassium phosphate buffer, 2.5µL EDTA, 2.5µL $MgCl_2$, 1µL $NADP^+$, 1 mL glucose 6 phosphate and 150µL crude enzyme. The mixture was incubated for 30 min at 37^{o}C. Optical density was measured by a spectrophotometer at 340 nm.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970). Gel was stained with Coomassie Brilliant Blue R-250 for the detection of protein bands.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Isolation of bacteria from industrial wastewater

In the present study, two bacterial isolates (lab designated names; 1f and 2b), out of 57 bacterial isolates obtained from industrial wastewater, were selected for further research work on the basis of their ability to tolerate and reduce tellurium. Physiochemical parameters (temperature, pH) of wastewater samples collected from an industrial area of Sheikhupura, Pakistan. The temperature of sample 1, 2 and 3 was 29ºC, 27ºC and 22ºC, respectively, while pH is 8, 8 and 7.0, respectively. The color of the samples were brown and black.

Table I: Morphological and biochemical characteristics of the bacterial isolates.

Characteristics	*S. epidermidis*	*S. lactis*
Shape	Round	Irregular
Size	3-4mm	1-2mm
Color	Yellow	Off-white
Elevation	Raised	Flat
Margin	Irregular	Irregular
Light transparency	Opaque	Translucent
Texture	Mucoid and shiny	Smooth and shiny
Gram staining	+ Cocci	+ Cocci
Catalase test	+	+
Manitol test	-	+
Pigment production	-	-

+: positive; -: negative

Heavy metal resistance

The MIC of tellurite for both isolates was upto l mM of $K_2Te_2O_3$. Both bacterial isolates showed resistance to heavy metals Cr^{+6} (0.3 mM), Cu^{+2} (0.5 mM), Cd^{+2} (0.3 mM), Zn^{+2} (0.3 mM), Pb^{+2} (0.1 mM), Ni^{+2} (0.5 mM) and As (0.3mM). Taylor (1999) reported that many strains of *E. coli* showed MIC >20 mM concentration of $K_2Te_2O_3$. Amoozegar *et al*. (2008) reported that MIC of many *Salinococcus* species showed 0.1 to 0.5 mM. Many halotolerant and halophilic microorganisms have

ability to tolerate heavy metals due to presence of high contents of cations as well as anions (Ventosa *et al*., 1998; Manzoor *et al*., 2016).

Characterization of tellurite resistant bacteria

Both of the isolates were circular in shape, Gram positive and showed the positive test for catalase positive. Isolate 2b has ability to ferment mannitol to produce yellow color, while (1f) isolate was unable to ferment mannitol. Morphological and biochemical characteristics of the isolates are given in Table 1. The partial sequences of 16S rRNA gene showed 99 and 97% homology with 16S rRNA sequence of *Staphylococcus epidermidis* and *Staphylococcus lactis*already submitted to NCBI database. Then the sequences were submitted to Genbank under the accession number of KY608969 and KY608970. Similarly, Fuentes *et al*. (2005) also isolated gram positive, cocci in clusters, which were non motile, non sporulated, and catalase positive which indicated genus *Staphylococcus*.

Optimum growth conditions

Physiological characteristics of bacterial isolates were determined. Both isolates showed maximum growth at 37ºC and pH of 7 (Fig. 1). The growth pattern of both isolates was evaluated in the presence and absence of tellurium (0.5mM) and it was observed that growth in the presence of metal was delayed as compared to the absence of metal (Fig. 2). Bacterial isolates from diverse locations supported diverse ranges of pH and temperature for their growth.

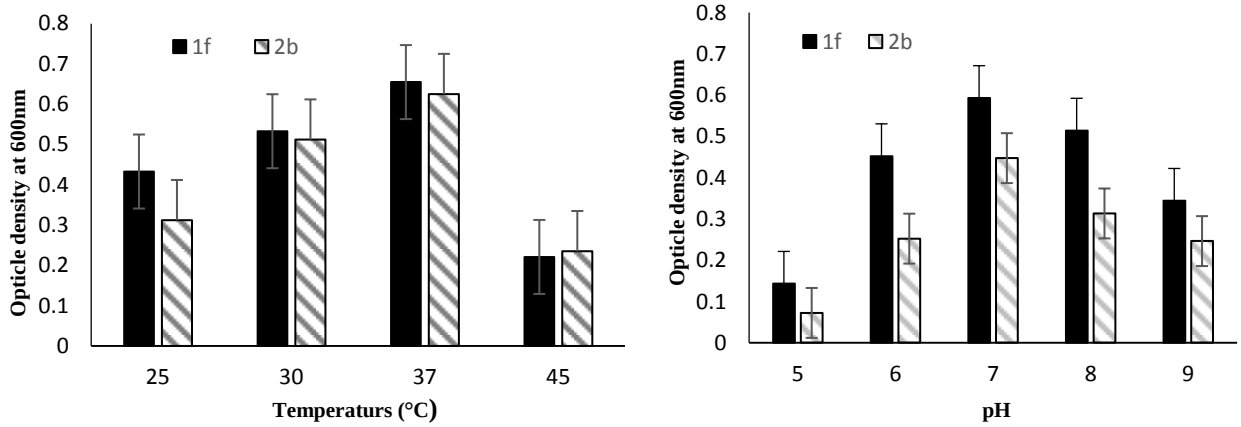

Figure 1. Effect of temperature and pH on the growth of bacterial isolates.

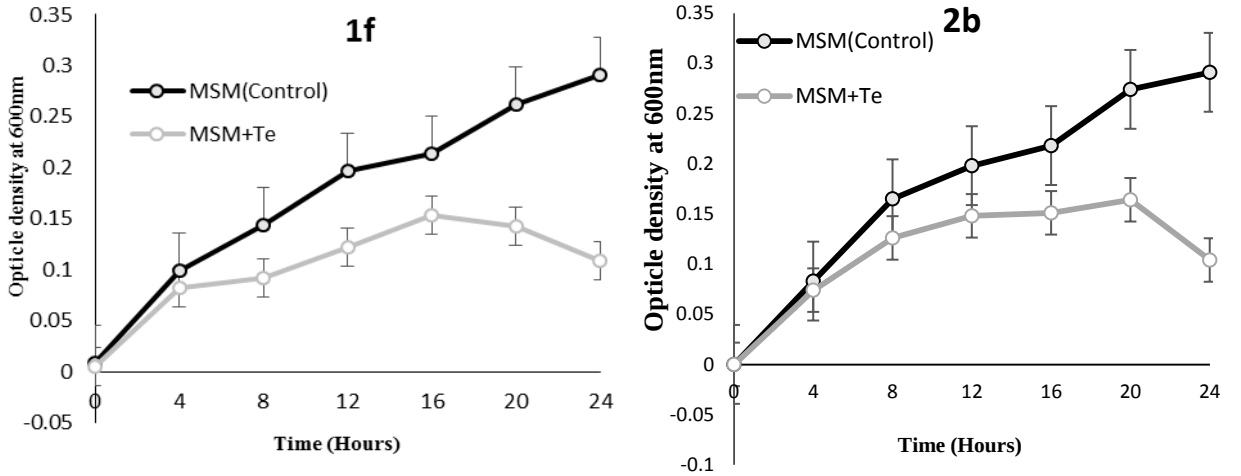

Figure 2. Growth of bacterial isolates (1f and 2b) in the presence (treated) and absence (control) of tellurite in MSM at 37ºC for different time period.

Amoozegar *et al*. (2008) reported that many halophilictellurite resistant bacteria showed maximum growth at 37ºC and pH 7. Pugin *et al*. (2014) showed that the growth curve of *S. aureus* was delayed in the presence of tellurium during lag phase, increased in lag phase (12-18 h), while after 18 h of incubation, bacterial growth rate was decreased rapidly. While in case of control, bacterial cells showed more growth after 8 h of incubation due to absence of metal stress.

Tellurite reduction potential of bacterial isolates

Tellurite reduction potential for both bacterial isolates was determined and it was found that bacterial isolates showed maximum reduction 98% (1f) and 97% (2b) after 24 h of incubation (Fig. 3). Castro *et al*. (2009) reported that low concentration of tellurium showed higher reduction potential of bacteria towards metal.

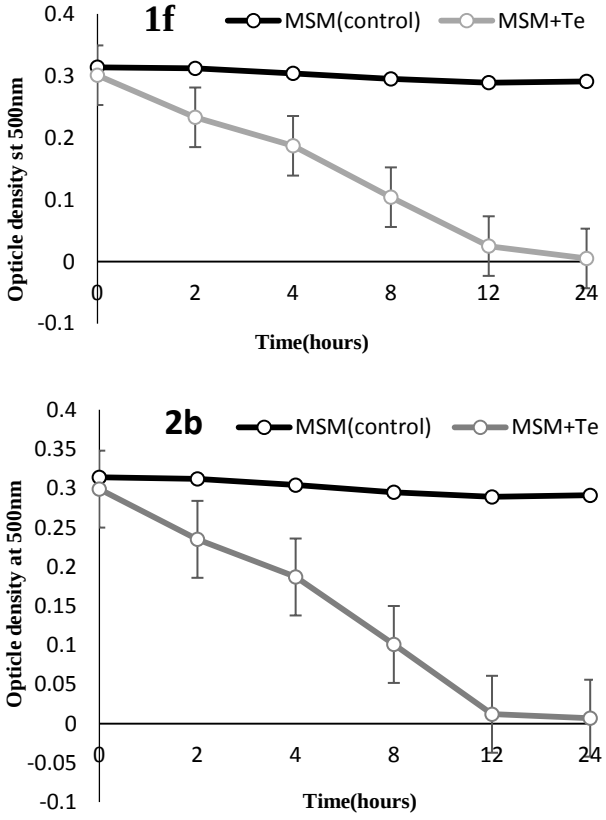

Figure 3. Tellurite reduction potential of bacterial isolates 1f and 2b in MSM for different time period.

Enzyme activity of G6PDH

Glucose 6 phosphate dehydrogenase (G6PDH) assay for both bacterial isolates was performed and both bacterial isolates showed maximum G6PDH relative activity in the presence of tellurite stress. Relative G6PDH activity of bacterial isolate (1f) was 279% while 2b showed 150% when compared to the respective control. This shows that both bacterial cells have ability to tolerate tellurite stress (Fig. 4). It indicates that NADPH level was increased in both bacterial isolates. G6PDH plays a vital role to keep normal bacterial cells under tellurite stress by reducing oxidized glutathione into its reduced form. Sandoval *et al*. (2011) reported that G6PDH content was also 80% in treated cells while their activities were low in control cells.

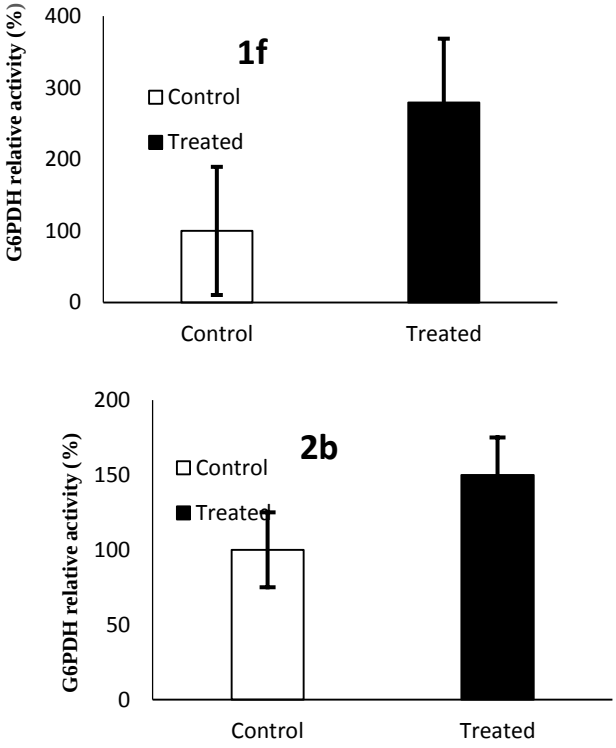

Figure 4. Glucose 6 phosphate dehydrogenase assay for bacterial isolates 1f and 2b.

SDS-PAGE analysis

The pattern of bacterial protein in the presence and absence of tellurite stress was determined by SDS-PAGE. It was found that the four protein bands (130, 95, 72 and 55 kDa) were observed both in the presence and absence of tellurite stress in case of 1f bacterial isolate. However, two protein bands, 170 kDa

and 43 kDa were not observed in 1f bacterial isolate which might be induced in the presence of tellurite stress. While two protein bands (95 and 55 kDa) were observed in control as well as in treated culture of 2b isolate (Fig. 5). Same proteins in the presence of tellurite stress with molecular masses ranging from 13 to 240 kDa have been reported (Chiong *et al*., 1988). However, majority of tellurite reducing bacteria presented molecular masses ranging from 55 to 60 kDa (Moscoso *et al*., 1998).

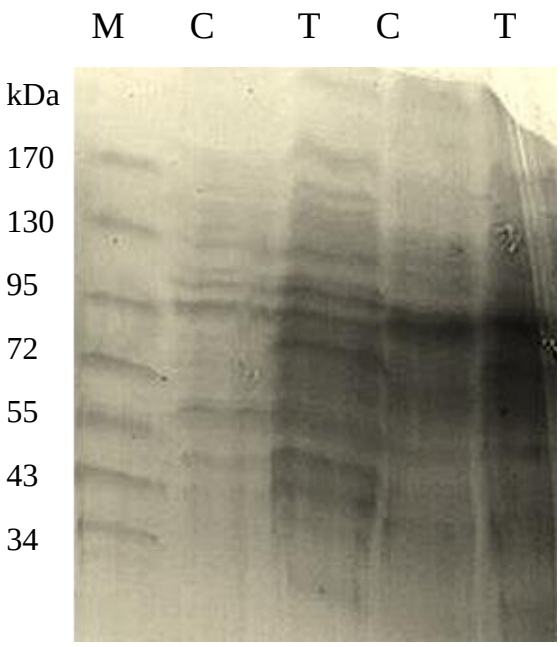

Figure 5. The protein profile of bacterial isolates (1f and 2b) through polyacrylamide gel electrophoresis; C represents control (without tellurite stress) and T represents treated (with tellurite stress); M is a protein ladder (prestained protein ladder # SMO671 Fermentas). The gel (12%) was stained with Coomassie blue G250

In conclusion, two bacterial isolates, *S. epidermidis* and *S. lactis*, were identified on the basis of biochemical tests and 16S rRNA sequence. The MIC of both bacterial strains showed fair growth up to 1mM of $K_2Te_2O_3$. Both strains showed maximum growth at 37ºC and pH of 7. The bacterial growth in the presence of metal ions was delayed as compared to the control. High tellurite reduction potential was shown 98% (1f) and 97% (2b) after 24 h of incubation. G6PDH activity was increasing upto 279% (1f) and 150% (2b) when compared to the respective control indicating the ability to tolerate tellurite stress. Differential pattern of bacterial proteins in tellurite stress was obtained by SDS-PAGE. The present study indicates that both bacterial strains have promising ability to reduce tellurite which can be exploited for the removal of tellurite from toxic wastewater released by the industries.

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Conflict of interest

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Pakistan.

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