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Antibacterial Efficacy of Manuka Honey against New Delhi Metallo-β-Lactamase Producing Gram Negative Bacteria Isolated from Blood Cultures

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Authors' Contribution
MUQ performed the experiments and wrote the manuscript. SJ and SS designed the study. UA contributed in data analysing. MFR and HE helped in samples collection and NS reviewed the manuscript.

Key words
Carbapenem resistance, NDM-1, Antibiotics and Manuka honey.

ABSTRACT

New Delhi metallo-β-lactamase (NDM-1) producing bacteria being responsible for multiple ailments, pose serious threat to human health. The NDM infections are constantly increasing worldwide particularly in sub-continent including Pakistan. These bacteria show resistance to wide range of broad spectrum antibiotics leaving the treatment options limited. Manuka honey is traditionally used for medical purposes including remedy of bacterial infection. However, scientific evidences for its antimicrobial effects particularly against NDM-1 producing bacteria are lacking. In addition to antibiotic profiling, the present study aimed at determining the *in vitro* anti- bacterial activity of Manuka honey against NDM-1 producing clinical isolates of Pakistan. A total of five carbapenem resistant bacterial species; *E. coli, K. pneumoniae, E. cloacae, A. baumannii* and *P. aeruginosa,* were isolated and identified using VITEK 2 compact system. Carbapenamase and metallo-β-lactamase (MBL) production by these isolates was confirmed by modified Hodge's test and disc potentiation method, respectively. The bla_{NDM-1}genewas amplified by PCR and sequenced. MIC of different antibiotics were determined by VITEK 2 system and antibacterial activity, MIC and MBC of manuka honey was performed by agar well diffusion assay and microbroth dilution assay respectively. All isolates (n=5) were carbapenamase and MBL producers andNDM-1 gene carrier. These isolates displayed significant resistance against commonly used antibiotics including carbapenem and colistin proved to be most effective drug. Manuka honey manifested significant antibacterial activity against all test isolates with almost similar zone of inhibition (7.4mm) except *E. cloacae*. Highest MIC and MBC of manuka honey was observed against *K. pneumoniae* (30%v/v) while lowest against *A. baumannii* (20%v/v). Here, we conclude that Manuka honey possess potent antibacterial activity and might be used as an alternative treatment option against NDM-1 producing bacterial species. However, further clinical trials are mandatory to corroborate our initial findings.

INTRODUCTION

Carbapenamases producing bacteria are extremely resistant to multiple drugs and spreading promptly across the globe (Pannaraj *et al*., 2015). These multi drug resistant (MDR) pathogens altogether, are accountable for high rate of morbidity and mortality throughout the world. The United Nation (UN) projected that in 2050, mortality rate due to MDR pathogens would exceed to 10 million per year (United Nations, 2015). New Delhi metallo-β-lactamase (NDM-1) is the emerging type of carbapenamase, discovered from a clinical isolate of *Escherichia coli* and *Klebsiella pneumonia* from the urine sample of a Swedish patient who had been hospitalized in New Delhi, India (Yong *et al*., 2009). Since then, it has rapidly spread worldwide probably via transposons and plasmids from one bacterium to another (Kumarasamy *et al*., 2010). The spread of NDM-1 positive pathogens has also been documented in Pakistan, where Qamar *et al*. (2015) reported the spread of NDM-1 producing bacteria in pediatric patients causing deaths in children. Recently, another study from Pakistan also stated the NDM-1 producing Gram negative bacteria responsible for 57% and 60% mortality in adults and neonatal patients

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respectively (Khan *et al*., 2016). NDM-1 producing pathogens produce antibiotic resistance not only against the β-lactam drugs but also against other different classes of antibiotics leaving limited treatment options (Munita and Arias, 2016). Consequently, therapeutic management of infections caused by NDM-1 producing bacteria has become a huge challenge for health authorities across the world.

Natural products have been traditionally used in the history for therapeutic purposes. The use of traditional medicine to treat infections is equally emphasized nowadays by World Health Organization (WHO) (Wachtel-Galor and Benzie, 2011). Manuka honey is mainly produced in New Zeeland and Australia by honey bees (*Apis mellifera*) foraging on the manuka or tea tree (*Leptospermum scoparium*) (Carter *et al*., 2016). It is of extreme medicinal importance because of its traditional use in the treatment of various bacterial and viral infections. Numerous studies have described the antibacterial activity of manuka honey against MDR pathogens. A study conducted in New Zeeland reported that manuka honey had significant antibacterial activity against *Streptococcus pyogenes*, *Shigella sonnii*, *Proteus mirabilis, Staphylococcus aureus* and *Listeria monocytogenes* (Balan *et al*., 2016). Jenkins *et al*. (2015) also determined the efficacy of manuka honey against *Pseudomonas aeruginosa* and *Burkholderia cepacia* which were inhibited at 4.5% w/v and 7.3% w/v, respectively. Similarly, Cooper *et al*. (2002) also revealed that *P. aeruginosa* isolated from burn patients were inhibited at the MIC of <10% v/v of manuka honey. Several factors such as acidic pH, high osmolality, hydrogen peroxide activity, stimulation of the immune response (tumor necrosis factor, interleukin), Unique Manuka Factor (UMF) or methylglyoxal compound (MGO) has been described being responsible for antibacterial activity of manuka honey (Franchini *et al*., 2007).

Albeit, the therapeutic potential of manuka honey has been investigated widely against a plethora of bacterial infections. However, to the best of our knowledge, no study was conducted so far, to explore the antibacterial activity of manuka honey against NDM-1 producing clinical isolates. Therefore, this study designed to determine the *in vitro* activity of manuka honey against NDM-1 producing bacterial species.

METHODOLOGY

Collection of bacterial strains

A total of five clinical carbapenem resistant bacterial strains; *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* were isolated from blood cultures of patients hospitalized in a tertiary care hospital of Lahore, Pakistan during September 2015.

Identification of the isolates

Bacterial strains were sub cultured on blood and MacConkey agar and plates were incubated at 37°C overnight aerobically. Isolates were identified based on their colony morphology and culture characteristics. Isolates were further confirmed using GN ID cards in VITEK 2 compact system (bioMerieux, France).

Phenotypic detection of carbapenamase

Carbapenamase detection was performed using modified Hodge's test (MHT) as per Clinical Laboratory Standard Institute (CLSI) 2015 guidelines. Briefly, carbapenem susceptible *E. coli* strain (ATCC 25922) was grown on Mueller Hinton agar (MHA) plate. Meropenem, a carbapenem antibiotic disc (10µg) was placed in the center of the plate and test isolates along with positive control (known clinical isolate of *K. pneumoniae*) and negative controls (*E. coli* ATCC 29522) were streaked from the edge of the disc towards periphery of the plate. Carbapenamase producing bacteria permitted the *E. coli* (ATCC 25922) to grow against the meropenem disc which leads to the cloverleaf like indentation, indicating positive results of MHT (Amjad *et al*., 2011).

Phenotypic detection of metallo-β-lactamases (MBLs)

MBLs detection was done by double disc potentiation method. MBLs producing bacteria require Zn^{+} as a cofactor and their activity can be lost by chelating agent like ethylene-diamine tetra acetic acid (EDTA). Concisely, test isolates were swabbed on MHA plate and two meropenem (MEM) (10µg) and two ertapenem (ETA) discs (10µg) were placed. 0.5M EDTA solution (10µL) was added on each meropenem and ertapenem disc. After incubation, results were interpreted with increase zone of inhibition (Yong *et al*., 2012).

Molecular identification of bla_{NDM-1} *gene*

The DNA was extracted from clinical isolates using commercially available DNA extraction kit (Tiagen, China). The presence of bla_{ND-1} gene in these isolates were assessed by PCR using specific primers (NDM-F 5′-ATGGAATTGCCCAATATTATGCAC-3′ and NDM-R 5′- TCAGCGCAGCTTGTCGGC-3′). The PCR conditions were; initial denaturation at 95°C for 5 min; second denaturation at 94°C for 1 min, annealing at 52°C for 40 sec, primary extension at 72°C for 1 min (cycles 35x) and final extension was done at 72°C for 10 min. After the PCR reaction, DNA was separated on agarose gel using gel electrophoresis to determine the presence and size of

the amplified DNA fragments.

DNA sequencing

Amplified PCR product was sequenced using forward and reverse primers (Macrogen, North Korea). The obtained sequences were analyzed by Basic Local Alignment Search Tool (BLAST) software available at National Center for Biotechnology and information (NCBI).

Minimum inhibitory concentration (MIC) of antibiotics

The MIC (µg/mL) of ampicillin/sulbactam, ticarcillin/clavulanic acid, piperacillin, cefuroxime, cefuroxime axetil, cefixime, ceftriaxone, cefepime, aztreonam, meropenem, levofloxacin, moxifloxacin, minocycline, tetracycline, tigecycline, chloramphenicol, colistin and trimethoprim against NDM positive isolates was performed using AST-XN05 cards in VITEK 2 compact system (bioMerieux, France). The results were interpreted as per CLSI 2015 breakpoints.

Agar well diffusion assay of Manuka honey

Antibacterial activity of undiluted Manuka honey (+20UMF) was evaluated by agar well diffusion assay against NDM-1 positive isolates adopted from punch plate assay (Boorn *et al*., 2010). Briefly, 0.5 McFarland bacterial suspension was inoculated on Mueller Hinton agar plate. A sterile 6 mm cork borer was used to make wells on each plate. Subsequently, 120 µL undiluted Manuka honey was poured in each well and plates were incubated at 37°C overnight. Zone of inhibition (mm) was measured by Vernier caliber. The assay was performed in triplicate

Determination of MIC and minimum bactericidal concentration (MBC) of Manuka honey

Microbroth dilution assay was employed to determine the MIC (%v/v) and MBC (%v/v) of the manuka honey (Wasihun and Kasa, 2016). Briefly, two isolated colonies of each strain were mixed in 20 mL of double strength Lysogeny broth (LB) medium in 50 mL of falcon tube and incubated at 37°C overnight. Each of the bacterial suspension was diluted to achieve 0.5 Mcfarland at OD of 0.07 at 600 nm spectrophotometrically. The suspension was further diluted 1:100 with double strength LB broth to achieve a final concentration of 1 x 10^5 CFU/mL. Briefly, serial dilutions of manuka honey (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50%) were prepared in sterile distilled deionized water and 100µL of each dilution was added in 96-wells, flat bottom micro titer plates (Thermo Fisher Scientific, UK). Subsequently, 100µL of bacterial suspension was added into each well. Negative control wells contained 100µL of LB broth only while positive control wells contained LB medium inoculated with bacteria suspension. Microtiter plate was incubated at 37°C overnight at shaking incubator and visually observed for the presence or absence of growth by comparing each well with negative and positive control wells. All the procedure was performed in triplicate. The MBC is defined as first dilution with no growth on agar plate. A 10µL sample was taken from the no visible growth wells of microtiter plate and was inoculated on the nutrient agar plates (Oxoid, UK) which were incubated aerobically at 37°C for 24 h. Plates were examined for cell viability. Any colonies that developed were scored as bacterial growth and no bacterial growth. All the procedures repeated in triplicate.

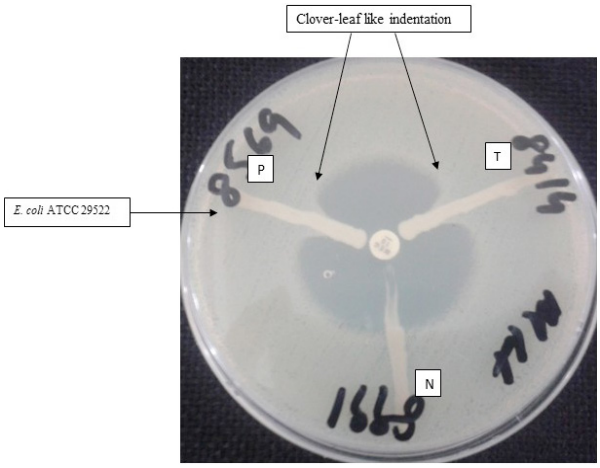

Fig. 1. Modified Hodge's test for phenotypic carabpenemase detection. T, test organism; P, positive control (*K. pneumoniae)*; N, negative control (*E. coli)*.

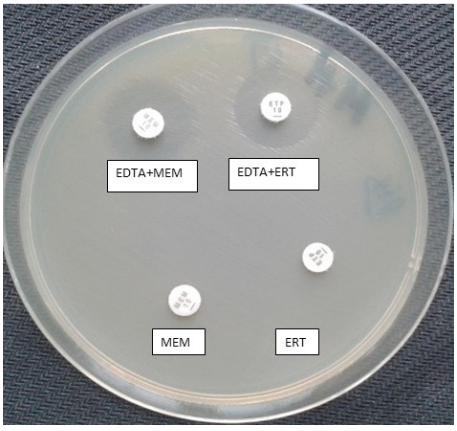

Fig. 2. Double disc potentiation method for the phenotypic detection of MBL producing bacteria. Positive results are shown by enlarged zone of inhibtion. MEM, meropenem disc; ETA, ertapenem disc; EDTA, ethylene-diamine tetra acetic acid.

Table I.- MIC (µg/mL) of different antibiotics againstNDM-1 positive bacteria (n=05) isolated from blood.

NDM-1 positive isolates	SAM	TCC	PIP	CXM	CXA	CFM	CRO	FEP	ATM	MEM	LEV	MXF	MNO	TE	TMP	C	TGC	CS
E. coli	>32	NT	>128	>64	>64	>4	>64	>64	>64	>16	>8	>8	4	>16	>16	16	<0.5	<0.5
K. pneumoniae	>32	NT	>128	>64	>64	>4	>64	>64	>64	>16	>8	>8	>16	>16	>16	>64	<0.5	<0.5
E. cloacae	NT	NT	>128	>64	>64	>4	>64	>64	>64	>16	>8	>8	>16	>16	>16	>64	1	<0.5
P. aeruginosa	>32	>128	>128	>64	>64	>4	>64	>64	NT	>16	>8	>8	>16	>16	>16	>64	1	<0.5
A. baumannii	>32	NT	>128	>64	>64	>4	>64	>64	>64	>16	>8	>8	4	>16	>16	>64	2	<0.5

NT, not tested; SAM, Ampicillin/Sulbactam; PIP, Piperacillin; CXM, Cefuroxime; CXA, Cefuroxime Axetil; CFM, Cefixime; CRO, Ceftriaxone; FEP, Cefepime; ATM, Aztreonam; MEM, Meropenem; LEV, Levofloxacin; MXF, Moxifloxacin; MNO, Minocycline; TE, Tetracycline; TMP, Trimethoprim; C, Chloramphenicol; TGC, Tigecycline; CS, Colistin.

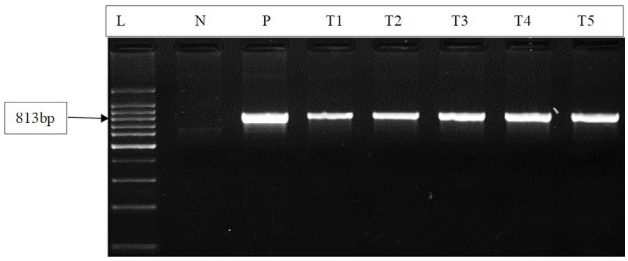

Fig. 3. PCR product of *bla*$_{NDM-1}$gene on 1.5% agarose gel. N, negative control; P, positive control; T1-T5, test sample; L, DNA ladder.

RESULTS

A total of five carbapenem resistant clinical isolates including *Escherichia coli* (n=1), *Klebsiella pneumoniae* (n=1), *Enterobacter cloacae* (n=1), *Acinetobacter baumannii* (n=1) and *Pseudomonas aeruginosa* (n=1) were isolated from the blood cultures of patients hospitalized in a tertiary care hospital of Lahore, Pakistan. The MHT revealed that all of the tested isolates (n=5) produce carbapenamases (Fig. 1). Similarly, it was concluded from double disc potentiation method that the strains under study were also MBL producers (Fig. 2). The results of these two aforementioned assays confirmed that all five bacterial isolates included in the study were resistant to carbapenem antibiotics. These findings were also confirmed at molecular level where all isolates showed the amplification of *bla*$_{NDM-1}$gene (Fig. 3) which encodes New Delhi metallo-β-lactamase. The isolates were further subjected to antibiotic profiling. The results revealed that all the isolates showed 100% resistance to β-lactam antibiotics including cephalosporins, aztreonam, carbapenems, β-lactam inhibitors, quinolones and macrolides. Moderate resistance was observed against minocycline. However, colistin with MIC of (≤0.5) proved to be most effective drug against NDM-1 producing isolates (Table I). The Agar well diffusion assay manifested the significant antimicrobial activity of manuka honey against tested clinical isolates. Antibacterial activity of manuka honey was almost similar against all tested isolates. However, maximum activity was noticed against *K. pneumonia* with zone of inhibition (mm) of 8.47mm ±0.82 followed by *E. coli* (7.3mm±0.47), *E. cloacae* (7.0mm±0.0), *P. aeruginosa* (7.6mm±0.4) and *A. baumannii* (7.5mm±0.0) (Table II, Fig. 4). MIC (%v/v) and MBC (%v/v) of the manuka honey against *E. coli*, *E. cloacae* and *P. aeruginosa* was 25% while *A. baumannii* and *K. penumoniae* were inhibited and killed at 20% and 30%, respectively (Table II).

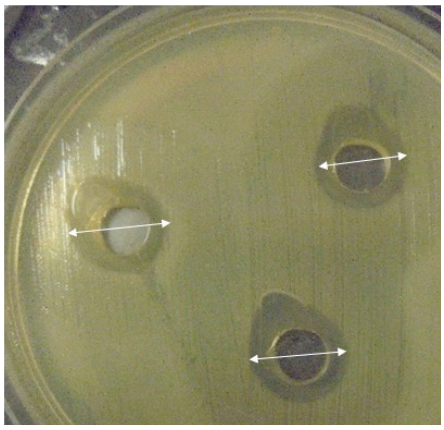

Fig. 4. Agar well diffusion assay for the determination of antibacterial activity of manuka honey. White arrows indicate the zone of inhibition (mm) produced by undiluted manuka honey against NDM-1 producing bacteria.

Table II.- Antibacterial activity of manuka honey against NDM-1 positive clinical isolates.

NDM-1 producing isolates	Agar well diffusion (mm±SD)	MIC (%v/v)	MBC (%v/v)
E. coli	7.3±0.4	25	25
K. pneumoniae	7.47±0.82	30	30
E. cloacae	8.0±0.0	25	25
P. aeruginosa	7.6±0.4	25	25
A. baumannii	7.5±0.0	20	20

DISCUSSION

The widespread and irrational use of antimicrobial agents leads to the development of multi-drug resistant pathogens (MDR). In clinical settings, MDR pathogens can be responsible for the prolong hospital stay, economic loss and devastating fatal consequences. Currently, NDM-1 producing bacteria are becoming a big challenge for public health care particularly in Indian subcontinent. Our study described that all the isolates (n=5) were carbapenamase and MBL producers that showed resistance against commonly used antibiotics including carbapenem. Manuka honey manifested significant antibacterial activity against all tested isolates. It was most effective against *K. pneumonia* while least effective against *A. baumannii*. We concluded that manuka honey possess potent antibacterial activity and might be used as an alternative treatment option against multidrug resistant pathogens.

In the present study, all the isolates have been found carbapenamase and MBL producers. The prevalence of MBL producing bacteria have been gradually increasing worldwide. In our previous study, we reported the isolation of MBL producing *E. cloacae* form pediatric patients in Pakistan (Qamar *et al*., 2015). Likewise, MBL producing Enterobacteriaceae were isolated from other Asian countries including China and Japan (Nishio *et al*., 2004). Moreover, an Indian study documented that 51% of the Gram-negative bacteria were MBL producing in their clinical settings (Azim *et al*., 2010).

In the present study, all NDM-1 producing bacteria manifested higher MIC (µg/mL) against commonly used antibiotics mainly carbapenems. However, colistin was found to be the most sensitive drug (Table I). These findings are in accordance with previous studies conducted in Pakistan by Qamar *et al*. (2015) and Khan *et al*. (2016) who also described the high drug resistance in NDM-1 producing *K. pneumoniae* and *E. coli* and colistin to be the most effective drug. Similarly, studies from other countries including India (Rahman *et al*., 2014), Bangladesh (Islam *et al*., 2012), United Kingdom (Kumarasamy *et al*., 2010) reporting that NDM producing *Enterobacteriaceae* are highly resistance to the β-lactam antibiotics while sensitive to colistin and tigecycline, corroborate our findings. The high resistance and spread of NDM producing bacteria particularly in Pakistan's clinical settings can be mainly justified by the irrational broad spectrum use of antibiotics, self-medication, use of imperial therapy and easy access of antibiotics at pharmacy corner. Other important factors are the bed sharing, poor health care facilities, unhygienic and sub-standard practices are very common (Hannan *et al*., 2013).

Manuka honey has been reported to contain antibacterial potential against most of the MDR pathogens. However, to our best knowledge, data is lacking regarding the antibacterial activity of manuka honey against NDM producing bacterial species. In the present study, manuka honey manifested significant antibacterial activity against all test NDM producing bacteria with almost similar zone of inhibition except for NDM-1 positive *E. cloacae* (Table II). As mentioned earlier, data is not available on the antibacterial activity of manuka honey against NDM positive isolates so far. However, a study from India reported the inhibition of Extended-spectrum β-lactamases (ESBL) producing *K. pneumoniae* by manuka honey (Pratibha and Manita, 2015). Simialary, studies from Irland and UK also documented the antibacterial effect of Manuka honey with high zone of inhibtion against MDR bacteria (Sherlock *et al*., 2010; Boateng and Diunase, 2015). In the present study, three NDM-1 producing pathogens were inhibited and killed at 25% v/v concentration of Manuka honey. Although no data available on the MIC and MBC of manuka honey against NDM-1 producing bacteria so far. However, recent study from India documented almost similar MIC and MBC of manuka honey against ESBL producing bacteria (Pratibha and Manita, 2015). Similalry, a stduy from UK reported the MIC (9.5%) and MBC (12%) of Mnauka honey against *P. aeruginosa* (Henriques *et al*., 2011). To summarize, manuka honey has been proved in this study as a promising bacteriocidal agents agaisnt NDM-1 producing bacterial species. Therefore, using honey for treatment NDM-1 infections may be worth checking.

In future, this study will help to evaluate the antibacterial efficacy of our local honeys *in vitro* as well *in vivo* in comparison with Manuka honey against various pathogens. Different animal models including rabbit, Guinee pig and mice can be subjected to monitor the pathogenesis of different NDM and MDR bacteria. Moreover, *in vivo* Manuka honey mediated immunological modulation, histological and hematological alterations can be investigated. Physiochemical analysis, host-microbe interaction and clinical trials involving animals and human of various types of honeys including Manuka honey can also be examined.

CONCLUSION

We concluded that Manuka honey has strong inhibitory effect against the NDM-1 produing bacteria. Therefore, it might be used as a treatment option against NDM produicng superbugs following the clinical trials.

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Statement of conflict of interest

All authors declared that no conflict of interest.

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