Transboundary and Emerging Diseases

RAPID COMMUNICATION

Detection of New Delhi Metallo-beta-Lactamase and Extended-Spectrum beta-Lactamase Genes in *Escherichia coli* Isolated from Mastitic Milk Samples

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Keywords:
metallo-beta-lactamase; *Escherichia coli*; extended-spectrum beta-lactamase; milk

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Received for publication April 6, 2013

doi:10.1111/tbed.12119

Summary

In this study, eight *Escherichia coli* isolates were obtained from milk samples of dairy cattle suffering from clinical/subclinical mastitis. Isolates were characterized for antimicrobial resistance traits and virulence genes. Results revealed that one isolate was harbouring New Delhi metallo-beta-lactamase gene (bla_{NDM}). Cloning and sequencing of the PCR amplicon confirmed the identity of the gene (GenBank accession no. KC769583) having 100% homology with bla_{NDM-5} (GenBank accession no. JN104597.1), and this isolate was susceptible to colistin, chloramphenicol and tetracycline only. Moreover, another isolate carried extended-spectrum beta-lactamase (ESBL) gene – bla_{CTX-M}, and all isolates possessed bla_{TEM} gene. Of the eight isolates, only one isolate was positive for shiga toxin gene (*stx2*), and none were harbouring *stx1* gene. Occurrence of New Delhi metallo-beta-lactamase (bla_{NDM}) in one *E. coli* isolate and ESBL genes in other isolates poses a potential threat to human health following possible entry and spread through food chain.

Introduction

Resistance due to metallo-beta-lactamases (MBLs) and extended-spectrum beta-lactamases (ESBLs) is emerging problems that compromise treatments of cases of bacterial infection. Among MBLs, New Delhi metallo-beta-lactamases (NDMs) are the recent ones that have been detected first in *Klebsiella pneumoniae* and *Escherichia coli* isolates (Yong et al., 2009). This was followed by numerous reports from many parts of the world (Pillai et al., 2011) including India (Kumarasamy et al., 2010; Manchanda et al., 2011). Of late evidence of detection of MBLs has started coming from animal associated isolates, for example, *Acinetobacter lwoffii* (Wang et al., 2012) and *E. coli* (Anonymous, 2012). Because enterobacteria carrying NDM gene are resistant to almost all antimicrobials with the exception of colistin and tigecycline (Kumarasamy et al., 2010), the presence of this gene among bacteria isolated from animal sources may pose a serious threat to human health as these bacteria may enter the food chain exposing general population to highly resistant bacteria. Similar concerns were also expressed previously regarding ESBLs (Perez et al., 2007).

In the present study, we report detection of New Delhi metallo-beta-lactamase (NDM) and ESBL genes in *E. coli*, isolated from milk samples of clinical/subclinical mastitis in cattle from West Bengal, India.

Materials and Methods

Bacterial strains

Eight *E. coli* isolates were obtained from milk samples collected from clinical/subclinical mastitis cases originating in adjoining areas of Kolkata during May 2012 to August 2012. Status of mastitis (clinical/subclinical) was determined (A. Singha, C. Guha, unpublished observation) by California mastitis test as described previously (Radostits et al., 2007).

Phenotypic characterization

Isolates were presumptively identified by their growth on eosine methylene blue agar (HiMedia, Mumbai, India) with typical greenish metallic sheen and four other biochemical tests (indole production, methyl red, Vogues Proskauer and citrate utilization) employing routine bacteriological techniques. Following presumptive identification, isolates were characterized by Phoenix™ 100 automated ID/AST system (Becton and Dickinson, Tuas Avenue, Singapore) for minimum inhibitory concentrations (MIC) of various antimicrobials employing Gram-negative combo panels (NMICID-55) (Becton Dickinson).

PCR for antimicrobial resistance and shiga toxin genes

PCRs for bla_{TEM} and $bla_{\text{CTX-M}}$ were performed with universal primers as described previously (Grobner et al., 2009) with suitable modifications. Briefly, reactions were optimized individually in 20 μl volumes with 10 pmol of each primer. Reactions were annealed at 50.5 and 55°C for *bla*-TEM and *bla*CTX-M, respectively. For $bla_{\text{NDM-1}}$, PCR was performed as described by Manchanda et al. (2011). In brief, PCR was standardized in 20 μl volumes with 10 pmol of forward and reverse primers. Primers were annealed at 59°C, and reaction was cycled for 35 times. PCR for shiga toxin genes (*stx1* and *stx2*) was optimized as described by Paton and Paton (1998).

PCR amplicons from all reactions were electrophoresed in 1.5% agarose gel and visualized under UV illumination (DNR MiniLumi gel documentation system).

Sequencing

$bla_{\text{NDM-1}}$ PCR amplicon was purified using AxyPrep™ PCR Cleanup Kit (Axygen, Union City, CA, USA) and was cloned using pGEM®-T kit (Promega, Madison, WI, USA) as per manufacturer's instruction. Sequencing was performed in an automated sequencer (Applied Biosystems, Grand Island, NY, USA) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) as per manufacturer's instructions. After sequencing results were obtained, homology searches were conducted using BLAST algorithm available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequence data were submitted to GenBank, and accession number was obtained.

Results and Discussion

Phenotypic characterization of isolates

Eight isolates that were presumptively identified as *E. coli* were confirmed by Phoenix™100 automated ID/AST system along with generation of antimicrobial susceptibility profiles. The resistance patterns revealed that isolate KOEC3 was resistant to almost all antimicrobials, and isolate KOEC6 was also moderately resistant to the antimicrobials tested (Table 1).

Minimum inhibitory concentrations data (Table 2) of these two isolates (KOEC3 and KOEC6) indicated that the isolate KOEC3 was susceptible to colistin and tetracycline only and was intermediately susceptible to chloramphenicol. Although *E. coli* isolates can on occasion contain different resistance markers (Garcia-Fernandez et al., 2008), isolates obtained from milk have not been reported previously with such widespread resistance, as in case of KOEC3.

Moreover, the isolate KOEC3 was identified by Phoenix 100 as 'potential carbapenemase' producer, while two isolates (KOEC3 and KOEC6) were ESBL producers (Table. 2).

Genotypic characterization

Characterization of isolates by PCR-targeting bla_{TEM} (universal primer), PCR-targeting $bla_{\text{CTX-M}}$ (universal primer) PCR-targeting $bla_{\text{NDM-1}}$ yielded positive signal(s) in all eight isolates for bla_{TEM}, in only one isolate for $bla_{\text{CTX-M}}$ and in one isolate for bla_{NDM}. PCRs resulted in amplification of products of expected molecular weights (851 bp for bla_{TEM}, 551 bp for line for $bla_{\text{CTX-M}}$ and 476 bp for $bla_{\text{NDM-1}}$).

In 2009, Shahid et al., 2009. documented the incidence of bla_{TEM} and $bla_{\text{CTX-M}}$ in members of enterobacteriaceae isolated from food samples and clinical samples in India in their study reporting the predominance of occurrence of bla_{TEM} followed by $bla_{\text{CTX-M}}$. In the present study, similar trend was also noticed.

On the other hand, detection of bla_{NDM} in an *E. coli* isolate from milk was rather unprecedented as was observed in the present study. To further confirm bla_{NDM}, the corresponding amplicon was cloned and sequenced, and a homology search was made using BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). The sequence was submitted to GenBank (accession no. KC769583). Result of sequencing identified a 476-bp product and BLAST search indicated 100% homology with $bla_{\text{NDM-5}}$ gene of *E. coli* (JN104597.1) and 99% homology with several sequences including $bla_{\text{NDM-4}}$ of *E. coli* (JQ348841.1) and $bla_{\text{NDM-1}}$ of *Acinetobacter baumannii* (KC404829.1, KC347597.1), *Roultella ornithinolytica* (JX680686.1), *K. pneumoniae* (KC178689.1), *Pseudomonas aeruginosa* (HF546976.1).

The isolate (KOEC3) bearing the bla_{NDM} gene was resistant to almost all antimicrobials (17 of 21) tested. However, susceptibility was observed for colistin (sensitive, MIC $\leq$ 0.5 μg ml^{-1}), tetracycline (sensitive, MIC $\leq$ 2 μg ml^{-1}) and chloramphenicol (intermediate, MIC 16 μg ml^{-1}) (Table 2). The MIC value of cefoperazone–sulbactam

Table 1. Resistance patterns of *E. coli* isolates isolated from milk[a]

	Isolates								Drugwise S-I-R patterns (%)		
Antimicrobials	KOEC1	KOEC2	KOEC3	KOEC4	KOEC5	KOEC6	KOEC7	KOEC8	S	I	R
Amikacin	S	S	R	S	S	S	S	S	87.5	0	12.5
Amoxicillin-Clavulanate	S	S	R	I	R	R	S	I	37.5	25	37.5
Ampicillin	S	S	R	R	R	R	S	R	37.5	0	62.5
Aztreonam	S	S	R	S	S	R	S	S	75	0	25
Cefazolin	S	S	R	S	R	R	S	S	62.5	0	37.5
Cefepime	S	S	R	S	S	R	S	S	75	0	25
Cefoperazone-Sulbactam[b]	≤0.5/8	≤0.5/8	>16/8	≤0.5/8	≤0.5/8	≤0.5/8	≤0.5/8	≤0.5/8	–	–	–
Cefotaxime	S	S	R	S	S	R	S	S	75	0	25
Cefoxitin	S	S	R	S	R	S	S	S	75	0	25
Ceftazidime	S	S	R	S	S	R	S	S	75	0	25
Chloramphenicol	S	S	I	R	S	S	I	R	50	25	25
Ciprofloxacin	S	S	R	R	S	R	S	R	50	0	50
Colistin[c]	S	S	S	S	S	S	S	S	100	0	0
Gentamicin	S	S	R	S	S	S	S	S	87.5	0	12.5
Imipenem	S	S	R	S	S	S	S	S	87.5	0	12.5
Levofloxacin	S	S	R	R	S	R	S	R	50	0	50
Meropenem	S	S	R	S	S	S	S	S	87.5	0	12.5
Piperacillin	S	S	R	R	S	R	S	R	50	0	50
Piperacillin-Tazobactam	S	S	R	S	S	S	S	S	87.5	0	12.5
Tetracycline	R	R	S	R	S	R	R	R	25	0	75
Trimethoprim-Sulfamethoxazole	S	S	R	R	S	R	S	R	50	0	50
Isolatewise S-I-R patterns (%)											
S	95	95	10	60	80	40	90	60			
I	0	0	5	5	0	0	5	5			
R	5	5	**85**	35	20	**60**	5	35			

Resistances to more than half the antimicrobials are indicated in boldface.

[a]SIR interpretations were as per Phoenix™ 100 except those of colistin and cefoperazone-sulbactam.

[b]Interpretation could not be inferred due to non-availability of appropriate guideline.

[c]Interpreted as per EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing, 2013).

could not be interpreted due to non-availability of an appropriate guideline (Table 2). The isolate was obtained from milk sample of a dairy cow suffering from mastitis, but it was not clear whether the same isolate was the primary cause of mastitis in this animal or not. Neither could we ascertain/trace the treatment history of the animal. We further screened the isolates for shiga toxin genes (*stx1* and *stx2*) as previously described by Paton and Paton (1998) with minor modifications. Results indicated that only one isolate (KOEC6 that was positive for ESBL as per Phoenix™ 100) was positive for *stx2*, and none of the isolates yielded amplification for *stx1*. This was in contrast to an earlier study reported by Momtaz et al. (2012) who reported *stx1* carrying *E. coli* from bovine mastitic milk. While studying a set of porcine *E. coli* isolates, Boerlin et al. (2005) reported statistical association between tetracycline resistance genes and *estA* virulence genes possibly due to some degree of physical linkages between virulence genes and those responsible for drug resistance (Martinez and Baquero, 2002). However, in our study due to limited number of isolates and low prevalence of shiga toxin genes, this hypothesis could not be tested. The isolate carrying $bla_{\text{NDM-1}}$ was not positive for either of the genes (*stx1* and *stx2*). While all isolates signalled positive amplification for bla_{TEM}, not all were identified as ESBL producer when tested in Phoenix™100. This was observed in 6 isolates. Although ESBL-producing *E. coli* were previously reported from animal sources (Hunter et al., 2010), occurrence of $bla_{\text{NDM-1}}$ gene was reported only recently in *Acinetobacter lwoffii* isolated from chicken rectal swab (Wang et al., 2012) and *E. coli* isolated from cat (Anonymous, 2012), and to the best of our knowledge, this is the first detection of bla_{NDM} gene in an *E. coli* isolate from milk samples of dairy cattle. Because the isolate was obtained from mastitis milk sample, detection of $bla_{\text{NDM-1}}$ was a matter of concern, because from milk, it might enter the food chain. As carbapenems are not in veterinary usage in India, it may be reasonably assumed that the gene has been horizontally acquired by the mastitis milk isolate from human or

Table 2. Minimum inhibitory concentration (MIC) values for two *Escherichia coli* isolates (KOEC3 and KOEC6)

	Isolates (MIC (μg ml^{-1})	
Antimicrobial	KOEC3	KOEC6
Amikacin	>32	≤8
Amoxicillin—clavulanate	>16/8	8/4
Ampicillin	>16	>16
Aztreonam	>16	>16
Cefazolin	>16	>16
Cefepime	>16	>16
Cefoperazone–sulbactam	>16/8	≤0.5/8
Cefotaxime	>32	>32
Cefoxitin	>16	≤4
Ceftazidime	>16	16
Chloramphenicol	16	8
Ciprofloxacin	>2	>2
Colistin	≤0.5	≤0.5
Gentamicin	>8	≤2
Imipenem	>8	≤1
Levofloxacin	>4	>4
Meropenem	>8	≤1
Piperacillin	>64	>64
Piperacillin–tazobactam	>64/4	≤4/4
Tetracycline	≤2	>8
Trimethoprim–sulfamethoxazole	>2/38	>2/38

environmental sources, or this may be due to a possible clonal spread from human/environmental origin. Considering the complexities of the aetiology of mastitis, it becomes fundamentally important to investigate the interplay and the causal relationships of the various microbial niches involved. Occurrences of multidrug-resistant bacteria in such samples could be reflective of accidental adaptation of a hitherto naive microorganism in a new target species, resulting in inadvertent establishment of infections of enhanced resistance with human health consequences.

Acknowledgement

Authors are thankful to the Director, ICAR RC for NEH Region, Umiam, Meghalaya, for providing necessary facilities for carrying out the research.

Conflict of interest

All authors declare 'no conflict of interest'.

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