

Emergence of *Escherichia coli*, Co-Producing NDM-1 and OXA-48 Carbapenemases, in Urinary Isolates, at a Tertiary Care Centre at Central India

ATUL KHAJURIA¹, ASHOK KUMAR PRAHARAJ², MAHADEVAN KUMAR³, NAVEEN GROVER⁴

ABSTRACT

Objective: To detect genes encoding carbapenem resistance in urinary isolates of *Escherichia coli* recovered from hospitalized patients in tertiary care centre in Pune, India.

Materials and Methods: From Jan 2012 to Dec 2012, a total of 300 consecutive non-duplicate (one isolate per patient) clinical isolates of *Escherichia coli* were recovered from urine cultures of hospitalized patients including hospital acquired infection cases admitted to the medical and surgical intensive care units. Polymerase chain reaction (PCR) assays and sequencing was used to determine the presence of beta-lactamase encoding genes. Conjugation experiments were performed to determine the transferability of beta-lactamase.

Results: All the isolates were completely resistant to the second and third generation cephalosporins tested as well as

carbapenems. All the isolates showed 100% susceptibility to tigecycline and colistin in vitro. Conjugation experiments demonstrated that *bla*_{NDM-1} was transferable via plasmid. All the isolates showed presence of *bla*_{NDM-1} and co-association of *bla*_{OXA-48} was 25/45(55%) of the isolates. Repetitive element based PCR (REP PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC PCR) and Randomly Amplified Polymorphic DNA (RAPD) revealed a diversity of six clonal types among *E.coli* isolates.

Conclusion: Co-production of NDM-1 with OXA-48 in urinary isolates of *E. coli* was detected for the first time in India. Transmission of plasmid carrying these resistant genes to other members of Enterobacteriaceae will increase incidence of multidrug resistance. Early detection of these genes will help in prevention and adequate infection control by limiting the spread of these organisms.

Keywords: Multidrug resistant, Carbapenem resistance, Conjugation, Beta-lactamase, Uropathogenic *E.coli*

INTRODUCTION

Beta-lactam antibiotics are one of the most frequently used antimicrobials in hospital settings used to treat infections caused by Gram negative bacteria. *Escherichia coli* is one of the most common pathogen of Enterobacteriaceae family responsible for nosocomial infections. However of lately due to the presence of extended-spectrum beta- lactamase (ESBL) and AmpC enzymes in these Gram negative bacilli, carbapenems have become the drug of choice to treat such infections. Incidence of multi drug resistance in organisms is increasing due to dissemination of resistance determinant genes mediated by gene cassettes in integrons, plasmids and transposons. Resistance to carbapenems due to carbapenemase production poses serious challenges in the treatment of such infections with pan-resistant phenotypes [1]. In this study molecular detection of resistant genes along with plasmid replicon typing of carbapenem resistant urinary isolates of *E.coli* was carried out and genes encoding both NDM-1 and OXA-48 in *E.coli* were detected.

MATERIALS AND METHODS

The Bacterial Clinical Isolates

The study was conducted after obtaining due approval from the institutional ethical committee. From Jan 2012 to Dec 2012, a total of 300 consecutive non-duplicate (one isolate per patient) clinical isolates of *E.coli* recovered from urine culture of hospitalized patients admitted to the medical and surgical intensive care units in 1000 bedded tertiary care hospital in Pune, India, were included in

the study. Collection of urine sample was done using strict aseptic precautions and was immediately processed without any delay. Urine culture was carried out on Cysteine Lactose Electrolyte Deficient (CLED) agar medium using calibrated standard loop. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques [2]. The organisms were identified up to the species level using VITEK-GNI cards (bioMérieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing and MIC Determination

Antibiotic sensitivity test was performed by standard Kirby Bauer disc diffusion technique as per the guidelines of the Clinical Laboratory Standards Institute (CLSI) with commercially available discs (Hi Media, Mumbai, India) on Mueller Hinton agar plates [3]. The antibiotics tested were as follows (potency in µg/disc): piperacillin (100), ticarcillin (75), piperacillin-tazobactam (100/10), ticarcillin-clavulanic acid (75/10), ceftazidime (30), cefotaxime (30), cefepime (30), cefoxitin (30), ceftriaxone (30), aztreonam (30), imipenem (10), meropenem (10), ertapenem (10), gentamicin (10), tobramycin (10), amikacin (30), netilmicin (30), ciprofloxacin (5), levofloxacin (5), lomefloxacin (10) and ofloxacin (5). *P. aeruginosa* ATCC 27853, *E.coli* ATCC 25922, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 were used as quality control strains. Minimum inhibitory concentrations (MIC) of antibiotics were determined by VITEK-2 AST-GN25 and AST-GN280 susceptibility cards in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations and manufacturers' instructions, except tigecycline and colistin, for

which the 2012 European Committee on Antimicrobial Susceptibility Testing break points were used[3,4]. MICs were further determined by the E-test (bioMérieux, Marcy l'Etoile, France).

Phenotypic Screening for the Carbapenemase Production

E.coli isolates with reduced susceptibility to meropenem and imipenem (diameter of zones of inhibition ≤ 13 mm) by disc diffusion method were screened for the production of carbapenemase. The phenotypic detection of the carbapenemase production was performed by the modified Hodge test by using a meropenem disc (10 μ g) as per CLSI guidelines [3]. For MHT *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were used as positive and negative controls, respectively. The screening of metallo-beta-lactamase production was performed by the double-disc synergy tests (DDST) and combined-disc synergy test (CDST) as described previously [5,6]. *K. pneumoniae* ATCC BAA-2146 and *P. aeruginosa* ATCC 27853 were used as positive and negative controls, respectively. MBL (IP/IPi) E-test was carried out to detect MBL as per manufacturer's instructions.

Molecular detection of the Beta-lactamase genes

DNA was extracted using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions. PCR-based detection of beta lactamase (ESBL) genes (bla_{CTXM} , bla_{SHV} , bla_{TEM} and bla_{OXA}), Ambler class B MBLs (bla_{IMP} , bla_{VIM} , bla_{SPM} , bla_{GIM} , bla_{SIM} and bla_{NDM-1}), Ambler class D (bla_{OXA-23} , bla_{OXA-24} and bla_{OXA48}) and for serine carbapenemases (bla_{KPC} , bla_{GES} and bla_{NMC}) were carried out on the isolates by using Gene Amp 9700 PCR System (Applied Biosystems, Singapore) [7]. PCR products were run on 1.5% agarose gel, stained with ethidium bromide visualized under UV light and photographed. The amplicons were purified using QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany)

DNA Sequencing and Sequence Analysis

Automated sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (<http://blast.ncbi.nlm.nih.gov>).

Conjugation Experiments

Transfer of resistance genes by conjugation was assayed by mating experiments in Luria-Bertani broth using *E. coli* urinary isolates (Parental strains) as donors and an azide-resistant *E. coli* J53 as the recipient strain using 1:10 ratio. The transconjugants were selected on Luria-Bertani agar with selection based on growth on agar in the presence of ceftazidime (30 μ g/ml) and sodium azide (100 μ g/ml). Plasmids were separated by co-electrophoresis on horizontal 0.5% agarose gel at 50 volts for 3 Hrs. The size of the plasmids were compared by co electrophoresis with plasmid of known sizes from *E.coli* (V517 and 39R861). Bands were visualized with UV transilluminator after staining with 0.05% ethidium bromide.

Strain Molecular Typing

Repetitive element based PCR (REP-PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) assays were performed as described to rapidly characterize bla_{NDM-1} positive *E. coli* strains recovered from patients [8]. Randomly Amplified Polymorphic DNA (RAPD) analysis was also carried out to detect relatedness of the strains [9].

Plasmid Analysis

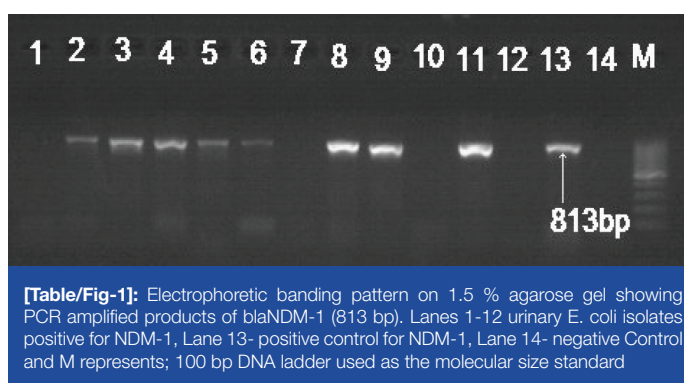
Plasmid from the parental strains and their transconjugants was extracted by using Qiagen plasmid mini kit (GmbH, Hilden, Germany) as per manufacturer's Instructions. Extracted plasmid DNA were subjected to plasmid based replicon incompatibility (Inc)

typing by using 18 pairs of primers to perform five multiplex and three single PCRs which recognized F, FIA, FIB, FIC, B/O, X, Y, N, P, W, T, A/C, HI1, HI2, I1-Ic, L/M, K and FII replicons as described previously [10]. Plasmid replicons were determined for the ESBL as well as carbapenemase producing clinical isolates.

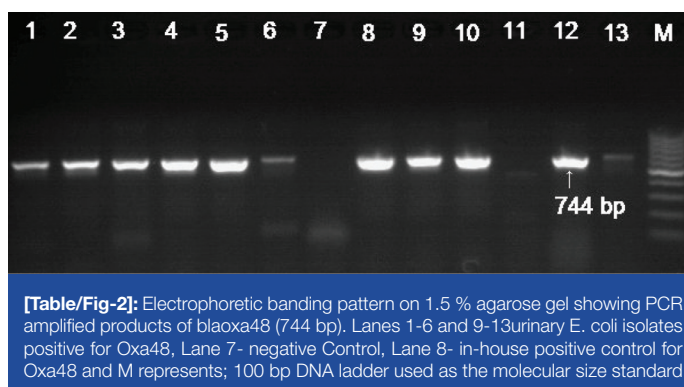
RESULT

Out of total 300 clinical urinary isolates of *Escherichia coli*, 45 were found to be carbapenem (imipenem, meropenem and ertapenem) resistant by the disk diffusion test and by e-test. These isolates showed resistance to other beta lactam antibiotics, aminoglycosides and quinolones tested. Carbapenemase production was confirmed by Modified Hodge test. Production of MBL was confirmed by positive DDST, CDST and MBL (IP/IPi) E-test method. All 45 carbapenem resistant isolates found to be positive for bla_{NDM-1} [Table/Fig-1] and 25 among these isolates found to be positive for bla_{OXA-48} [Table/Fig-2]. Antibiogram of these 25 isolates are depicted in [Table/Fig-3]. The PCR results were validated by sequencing. Overall $bla_{CTX-M-15}$ was the commonest genotype 38/45 (84%) followed by bla_{TEM} 32/45(71%), bla_{SHV} 28/45(62%) and bla_{OXA} 19/45(42%) either alone or in combination [Table/fig-4]. Based on sequencing it was confirmed that bla_{TEM-1} , bla_{SHV-5} , bla_{SHV-11} , bla_{SHV-12} , bla_{SHV-28} , $bla_{CTX-M-15}$, bla_{OXA-1} , bla_{OXA-2} , bla_{OXA-9} and bla_{OXA-10} are the dominant ESBLs among these carbapenem resistant strains in our study. Conjugation experiments indicated that bla_{NDM-1} was transferable via a plasmid along with other beta lactamase genes carried on other plasmids. Plasmid profiling of the isolates showed that bla_{NDM-1} was carried on plasmids ranging in sizes from 70 to 120 kb and bla_{OXA-48} was carried on 50 kb size plasmids.

REP-PCR, ERIC-PCR and RAPD assays confirmed presence of 6 clones as per banding pattern. Plasmids purified from the clinical isolates were typed by PCR based replicon typing. IncFIA, IncFIB, IncFIC replicons were associated with bla_{TEM-1} . Majority of bla_{SHV} showed association with multiple replicons (either IncFII, IncFIB or IncFIA, IncFIB), three isolates showed single replicon association (IncFIC). The bla_{NDM-1} gene in *E.coli* was located on IncA/C plasmid. The bla_{OXA-48} carried on plasmids belonging to Inc L/M replicons. $bla_{CTX-M-15}$ was associated with multiple replicons of plasmid (IncFIA, IncFIB). The bla_{OXA} identified on plasmids was associated with IncP and IncW replicons [Table/Fig-5].



[Table/Fig-1]: Electrophoretic banding pattern on 1.5 % agarose gel showing PCR amplified products of bla_{NDM-1} (813 bp). Lanes 1-12 urinary *E. coli* isolates positive for NDM-1, Lane 13- positive control for NDM-1, Lane 14- negative Control and M represents; 100 bp DNA ladder used as the molecular size standard



[Table/Fig-2]: Electrophoretic banding pattern on 1.5 % agarose gel showing PCR amplified products of bla_{OXA48} (744 bp). Lanes 1-6 and 9-13 urinary *E. coli* isolates positive for Oxa48, Lane 7- negative Control, Lane 8- in-house positive control for Oxa48 and M represents; 100 bp DNA ladder used as the molecular size standard

Antibiotic used	MIC µg/ml
amikacin [AMK]	64
ampicillin[AMP]	128
cefepime [CPM]	64
ceftazidime [CAZ]	128
cefuroxime [CFR]	64
ciprofloxacin[CIP]	4
Cefotaxime[CTX]	128
ertapenem[ETP]	8
gentamicin [GEN]	16
imipenem [IPM]	64
levofloxacin [LEV]	8
meropenem [MEM]	64
moxifloxacin[MXF]	8
piperacillin[PIP]	128
tobramycin [TOB]	16
colistin [CST]	<0.5
tigecycline [TGC]	< 2

[Table/Fig-3]: Showing MICs of urinary *E. coli* isolates against antibiotic used co-producing NDM-1 and OXA-48 carbapenemases (n=25)

Name of gene	Present	Percentage
NDM-1	45	100%
OXA 48	25	55%
CTX-M-15	38	84%
TEM-1	32	71%
SHV type ESBL SHV5, SHV11, SHV12, SHV28	28	62%
OXA type ESBL OXA-1, OXA-2, OXA-9, OXA10	19	42%

[Table/Fig-4]: Showing distribution of Beta Lactamase genes in multidrug resistant urinary *E. coli* isolates (n=45)

I.D	TEM	SHV	CTX-M	OXA	OXA- 48	NDM-1
UC10	FIB	FII,FIB	FIA,FIB	P	L/M	A/C
UC53	FIC	NG	FIA,FIB	NG	L/M	A/C
UC66	FIB	FIA,FIB	FIA,FIB	P	L/M	A/C
UC251	FIC	FII,FIB	FIA,FIB	NG	L/M	A/C
UC320	FIA	FII,FIB	FIA,FIB	P	L/M	A/C
UC405	FIB	NG	FIA,FIB	W	L/M	A/C
UC480	FIA	FIA,FIB	FIA,FIB	NG	L/M	A/C
UC1387	FIC	FIC	FIA,FIB	NG	L/M	A/C
UC1445	NG	FIC	FIA,FIB	P	L/M	A/C
UC1632	NG	FIA,FIB	FIA,FIB	NG	L/M	A/C
UC1862	FIB	FII,FIB	FIA,FIB	NG	L/M	A/C
UC1880	FIC	NG	FIA,FIB	W	L/M	A/C
UC2044	FIC	FII,FIB	FIA,FIB	W	L/M	A/C
UC2330	FIA	FIA,FIB	FIA,FIB	NG	L/M	A/C
UC2454	NG	FIC	FIA,FIB	NG	L/M	A/C
UC2632	FIB	FII,FIB	FIA,FIB	W	L/M	A/C
UC2778	FIC	FII,FIB	FIA,FIB	P	L/M	A/C
UC2981	FIA	FII,FIB	FIA,FIB	P	L/M	A/C
UC3028	FIB	FII,FIB	FIA,FIB	NG	L/M	A/C
UC3239	FIA	NG	FIA,FIB	NG	L/M	A/C
UC3521	FIC	FIA,FIB	FIA,FIB	NG	L/M	A/C
UC3649	FIA	FIA,FIB	FIA,FIB	P	L/M	A/C
UC3741	NG	FIA,FIB	FIA,FIB	NG	L/M	A/C
UC4008	FIC	NG	FIA,FIB	NG	L/M	A/C
UC4330	FIB	NG	FIA,FIB	NG	L/M	A/C

[Table/Fig-5]: Showing replicon typing in multidrug resistant *E. coli* isolates co-producing NDM-1 and OXA-48 carbapenemases

DISCUSSION

E. coli is a common cause of community-acquired and health-care-acquired infections. Carbapenems are being increasingly used to treat infections due to multi drug resistant Enterobacteriaceae and sometimes empirically. This has got a major impact in the emergence of multi drug resistance which can be easily transmitted from one species to another by transferable elements such as plasmids. MIC values for imipenem, meropenem and ertapenem ranged from 8-64 µg/ml. Strains found to harbor both *bla*_{NDM-1} and *bla*_{OXA-48} showed higher MICs against carbapenems (64 µg/ml) as compared to MICs (8-16 µg/ml) showed by strains harboring *bla*_{NDM-1} only. Isolates were found to be susceptible to tigecycline and colistin as per MIC breakpoints. In this study, 45 (100%) *bla*_{NDM-1} positive *E. coli* isolates showed positive results from the modified Hodge test while finding from Castanheira M et al., reported the occurrence of weakly positive results for the modified Hodge test in the detection of NDM-1 producing Enterobacteriaceae[11]. There was a 100% correlation with positive DDST, CDST and MBL (IP/IPI) E-test method with the presence of NDM-1 in these clinical isolates as detected by PCR. The overall co-presence of *bla*_{OXA-48} and *bla*_{NDM-1} among *E. coli* in our study was found to be (25/300) 8.3%. Among ESBL *bla*_{CTX-M-15} was the commonest genotype 38/45 (84%) followed by *bla*_{TEM} 32/45(71%) *bla*_{SHV} 28/45 (62%) and *bla*_{OXA} 19/45(42%) either alone or in combination in the *bla*_{NDM-1} producing *E. coli*. Previous studies from India had reported the presence of TEM-1, CTX-M-15, SHV-1, SHV-12, DHA and CMY-2 and in the NDM-1 producing Enterobacteriaceae [12,13]. While findings from other studies from abroad had showed the presence of *bla*_{CTX-M-15}, *bla*_{TEM-1}, *bla*_{SHV-28}, *bla*_{SHV-11}, and *bla*_{CMY-6} in the *bla*_{NDM-1} possessing Enterobacteriaceae [14,15]. Though, the strain remains sensitive for tigecycline in vitro but it is not recommended for use in UTI infections. Colistin is the main stay of therapy.

CONCLUSION

Both bla_{NDM-1} and bla_{OXA-48} resulted in higher MICs against carbapenems (64 µg/ml) than presence of bla_{NDM-1} alone (>8-32µg/ml). This must be extremely worrisome, as dissemination of plasmids carrying resistant determinant genes from one species to another makes organism refractory to the common antibiotics used in clinical practice. Here we report the co-presence of NDM-1 with OXA-48 producing *E.coli* in urine culture from a tertiary care centre in central India. Early detection of these resistant determinant genes by molecular methods is essential in limiting the spread of infection due to these organisms.

REFERENCES

- [1] Livermore DM and Woodford N. *The beta-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter*. *Trends Microbiol*. 2006. 14:413-420.
- [2] Collee JG, Miles RS, Wan B. Tests for the identification of bacteria. In: Collee JG, Fraser AG, Marmion BP, Simmons A editor. *Mackie and Mc Cartney Practical Medical Microbiology*. 14th ed. Edinburgh: Churchill Livingstone; 1996; p. 131-150
- [3] Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; twenty second Informational Supplement M100-S22*. CLSI, Wayne, PA, USA, 2012.
- [4] European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters (Version 2, January 1, 2012). http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2_0_120221.pdf
- [5] Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge Test and the Imipenem-EDTA Double-Disk Synergy Test for differentiating metallo-β-lactamase producing Isolates of *Pseudomonas* spp. And *Acinetobacter* spp. *J Clin Microbiol*. 2003; 41:4623-6.
- [6] Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem EDTA disc method for differentiation of metallo beta lactamase producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2002;40:3798-801.
- [7] Kumarasamy K, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis*. 2010; 10:597-602.
- [8] Versalovic J, Koeuth T and Lupski J R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res*. 1991;19:6823-31.
- [9] Vogel L, Jones G, Triep S, Koek A and Dijkshoorn L. *RAPD* typing of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Pseudomonas aeruginosa* isolates using standardized reagents. *Clin. Microbiol.Infect*.1999; 5:270-76.
- [10] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL and Threlfall EJ. Identification of plasmids by PCR-based replicon typing methods. *J. Microbiol*. 2005;63: 219-28.
- [11] Castanheira M, Deshpande LM, Mathai D, Bell JM, Jones RN and Mendes RE. Early dissemination of NDM-1- and OXA-181-producing Enterobacteriaceae in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program, 2006-2007. *Antimicrob Agents Chemother*. 2011; 55:1274-78.
- [12] Lascols C, Hackel M, Marshall SH, Hujer AM, Bouchillon S, Badal R, et al. The increasing prevalence and the dissemination of NDM-1 metallo-β-lactamase in India: data from the SMART study (2009). *J Antimicrob Chemother*. 2011; 66:1992-97.
- [13] Khan AU, Nordmann P. NDM-1-producing Enterobacter cloacae and *Klebsiella pneumoniae* in diabetic foot ulcers in India. *J Med Microbiol*. 2012; 61:454-56.
- [14] Samuelsen Ø, Thilesen CM, Heggelund L, Vada AN, Kummel A and Sundsfjord A. Identification of NDM-1-producing Enterobacteriaceae in Norway. *J Antimicrob Chemother*. 2011;66:670-2.
- [15] Poirel L, Revathi G, Bernabeu S and Nordmann P. Detection of NDM- 1-producing *Klebsiella pneumoniae* in Kenya. *Antimicrob Agents Chemother* 2011; 55: 934-36.

PARTICULARS OF CONTRIBUTORS:

1. PhD Scholar, Department of Microbiology, Armed Forces Medical College Pune, India.
2. PhD Scholar, Department of Microbiology, Armed Forces Medical College Pune, India.
3. Post Graduate, Department of Microbiology, Armed Forces Medical College Pune, India.
4. Post Graduate, Department of Microbiology, Armed Forces Medical College Pune, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Atul Khajuria,
Department of Microbiology, AIIMS, Bhubaneswar-751019, Odisha, India.
Phone: 07751958681, E-mail: Atulkhajuria83@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Oct 26, 2013**
Date of Peer Review: **Jan 20, 2014**
Date of Acceptance: **Mar 05, 2014**
Date of Publishing: **Jun 20, 2014**