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ORIGINAL ARTICLE

NDM 1 Gene Carrying Gram negative Bacteria Isolated from Rats Captured from some Poultry Houses in Nigeria

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SUMMARY

The New Delhi metallo-beta lactamase (NDM-1) bearing pathogens pose serious public health threat because they are usually resistant to virtually other useful antibiotics. Constant monitoring and prompt detection of such pathogens are keys to effective prevention and control of their spread among other animal and human pathogens. In this study, we screened 56 Gram negative bacteria comprising: 3 isolates of *Enterobacter ludwigii*, 30 *Pseudomonas aeruginosa*, 22 *Proteus mirabilis*, and 1 *Aeromonas caviae* isolated from oral cavity and rectum of rats captured from commercial poultry houses in Ibadan, Oyo State, Nigeria that were resistant to at least three of or all of: ceftazidime, ceftriaxone, amikacin and cefepime at 32µg/mL breakpoint for the presence of *bla* NDM-1, using PCR assay. The bacteria isolates were identified based on standard bacteriological procedures and by 16S PCR identification method. The isolates were screened to find out the possible role of rats in the dissemination and the public health importance of the species to poultry and ultimately to humans. A total of 5.4% (3/56) of the Gram negative bacteria from rats screened from the studied area carried the NDM-1 gene. The results from this work indicated that rat may serve as agent for disseminating the gene to poultry and human pathogens. Rat control within the poultry houses and human households should be given important consideration to achieve effective prevention and control of spread of these organisms that may be of public health importance.

Key words: Rats, poultry, New Delhi metallo-beta lactamase (NDM-1).

INTRODUCTION

New Delhi metallo-beta- lactamase-1 (NDM-1) which was first described in 2009 from *Klebsiella pneumoniae* and *Escherichia coli* isolated from a Swedish patient that earlier received a medical care in India and it belonged to class B of the metallo beta lactamase (Yong et al., 2009; Kumarasamy et al., 2010). New Delhi metallo-beta-lactamse-1 (NDM-1) producing bacteria constitute a global public health problem due to their resistance to most antibiotics including virtually all β-lactams (carbapenems inclusive), with the exception of colistin and tetracycline

(Kumarasamy et al., 2010; Poirel et al., 2010; Mulvey et al., 2011). Their public health threats in terms of the multidrug resistant nature of pathogens bearing the gene has taken a global dimensions because of rapid dissemination to different parts of the world, following its initial description (Walsh, 2010). It has been reported in different parts of the world such as: United Kingdom, India, Pakistan, the Sultanate of Oman, across Europe; Netherlands, Australia, France; Africa and Canada (Yong et al., 2009; Struelens et al., 2010; Poirel et al., 2010; Leverstein-Van Hall et al., 2010; Nordmann et al., 2011; Walsh and Toleman, 2012, Patel and Bonomo, 2013; Manenzhe et al., 2015). Due to the public health importance of pathogens carrying this gene and their rapid disseminations across the world, its continuous monitoring and rapid detection is considered very important to check their spread and in the formulation of effective infection control (Farzana et al., 2013).

Recently, 30 multidrug resistant *Pseudomonas aeruginosa* that were found be resistant at various percentages to 10 commonly used antibiotics including; 100% resistance to ceftazidime and ceftriaxone, 96.66% resistance to amikacin and 76.66% resistance to cefepime among the other antibiotics like kanamycin, ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and levofloxacin at breakpoint concentrations, were identified from rats captured from some poultry houses in Ibadan, Oyo state Nigeria (Ogunleye et al., 2015). In the current work, we therefore screened 56 Gram negative bacteria comprising: 3 *Enterobacter ludwigii*, the 30 *Pseudomonas aeruginosa,* 22 *Proteus mirabilis*, and 1 *Aeromonas caviae* isolated from oral cavity and rectum of rats captured from the same commercial poultry houses in Ibadan, Oyo State, Nigeria. Based on their resistance to at least three of the following antibiotics: ceftazidime, ceftriaxone, amikacin and cefepime at 32µg/mL breakpoint, the presence of *bla* NDM-1 was investigated to find out if rats could serve as possible reservoirs for the dissemination of the gene to poultry pathogens and ultimately to human pathogenic bacteria in the study area.

MATERIALS AND METHODS

Bacteria isolates

The bacteria screened for the presence of NDM 1 genes included 56 Gram negative bacteria comprising 3 *Enterobacter ludwigii*, 30 *Pseudomonas aeruginosa*, 22 *Proteus mirabilis*, 1 *Aeromonas cavie* isolated from the oral cavity and rectum of rats captured from commercial poultry houses in Ibadan, Oyo State, Nigeria. The bacterial isolates were identified according to standard bacteriological procedures (Barrow and Feltham, 2004; Garcia and Isenberg, 2007). Their identities were further confirmed with Oxoid Microbact GNB 24E® (MB24E) and accompanying computer software package (Oxoid Microbact®) 2000 version 2.03 according to the manufacturers procedures and through 16S ribosomal RNA PCR identification procedure. They were screened for presence of NDM 1 gene based on their resistance to at least three of or all of the following antibiotics: ceftazidime, ceftriaxone, amikacin and cefepime at 32µg/mL breakpoint.

16S RNA Identification of the Gram negative bacteria

The 16S ribosomal RNA identification of the 56 isolates were performed as previously described by Weisburg and his co-workers (Weisburg et al., 1991) as modified by (Ogunleye *et al.,* 2015).

Chromosomal DNA were produced from the 56 isolates by heating the LB broth cultures at 99^0C for 15minutes. A 100µl of the boiled isolates were mixed with equal volume of PCR grade water, 1 µl of the mixture was used as DNA template in a 50

µl reaction. The DNA was amplified using QS PCR reagents(New England Bio labs) containing 1µM of fD2= 5'AGATTTGATCATGGCTCAG3' and rP1 = 5'ACGGCTACCTTGTTACGACTT3', including 10 µl QS buffer, 1 µl dNTPs, 0.25 µl fD1, 0.25 µl rP1, 0.5 µl QS enzyme, 10 µl QS enhancer and 27 µl PCR water, using the PCR programme: initial denaturation step at 98^0C for 30 seconds, followed by 35 cycles of DNA denaturation at 98^0C for 10 seconds, primers annealing at 55^0C for 30seconds, primers extension at 72^0C for 1minutes 15seconds followed by final extension at 72^0C for 7minutes. The amplified products were resolved with precasted E- gel in an Electrophoresis unit (Life Technologies).

The amplified products were purified with Qiagen kits and sequenced at Iowa State University DNA sequencing facilities (Ames, IA, USA). The identities of the sequenced products were analysed by using BLASTN 2.2.31+ as described by Zhang and co-workers (Zhang et al., 2000).

Determination of Resistance to ceftazidime, ceftriaxone, cefepime and amikacin

The isolates were grown aerobically at breakpoint concentrations of 32µg/mL each for ceftazidime, ceftriaxone, amikacin, and cefepime (all from SIGMA- ALDRICH) according to standard method (CLSI, 2009). Resistance was ascribed if flocculent growth was observed after 16 hours of aerobic growth at 37^0C.

NDM-1 gene screening

The 56- Gram negative bacteria shown in table 1 were screened for the presence of bla NDM-1 by PCR as earlier described with some modifications (Chen et al., 2011). All the isolates were screened with primers NDM- 1 F (5′ -ATGGAATTGCCCAATATTAT-3′) and NDM- 1 R-(5′ -TCAGCGCAGCTTGTCGGCCA-3′). The primers were made available by Dr Steve Carlson of the Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames, USA, with the expected amplicon size of 814bp. Chromosomal DNA was produced from each of the 56 isolates by heating 100µl LB broth cultures at 99^0C for 15minutes using the thermal cycler. A 100µl of the boiled isolates were subsequently mixed with equal volume of PCR grade water, 1 µl of the mixture was used as DNA template in a 50 µl reaction. The DNA was amplified using QS PCR reagents(New England Bio labs) containing 1µM of each forward and reverse primers including 10 µl QS buffer, 1 µl dNTPs, 0.25 µl NDM 1- F, 0.25 µl NDM-1 R, 0.5 µl QS enzyme, 10 µl QS enhancer and 27 µl PCR water, using the PCR protocol: initial denaturation at 98^0C for 30 seconds, and 35 cycles consisting of denaturation at 98^0C for 10seconds, annealing at 55^0C for 30secondss, and extension at 72^0C for 1minute 15seconds and a final extension at 72^0C for 7minutes. The synthesized DNA manufactured by Genscript^R with the Oligosequence shown in Figure 2 was used as positive control. The amplified products were resolved with precast E- gel in an Electrophoresis unit (Life Technologies) in the Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames, United States of America.

RESULTS AND DISCUSSION

From the 56 Gram negative bacteria screened for the presence of NDM-1 gene, 5.4% (3/56) including; 2 *Proteus mirabilis* and 1 *Enterobacter lugwigii* were positive with the expected band of about 814bp (Table 2). TABLE I showed the resistance patterns of the Gram negative bacteria to the four antibiotics of interest screened. Figures 1 shows the gel picture of two of the amplified NDM-1 gene in isolates A15nlf

TABLE I: Susceptibility of 56 bacterial isolates to four antibiotics

Isolate	16s rna identity	Source	Ceftaz	Ceftria	amik	cefep
B5lf	*Enterobacter ludwigi*	Rat	R	R	R	R
B26lf	*Enterobacter ludwigi*	Rat	R	R	R	R
B54lf	*Ent lugwigi*	Rat	R	R	R	R
B30nlf	*Aeromonas cavie*	Rat	R	R	R	R
B34nlf	*P.aeruginosa*	Rat	R	R	R	R
B26nlf	*P.aeruginosa*	Rat	R	R	R	R
B54nlf	*P.aeruginosa*	Rat	R	R	R	R
B13nlf	*P.aeruginosa*	Rat	R	R	R	R
A24nlf	*P.aeruginosa*	Rat	R	R	R	R
A54nlf	*P.aeruginosa*	Rat	R	R	R	R
A27nlf	*P.aeruginosa*	Rat	R	R	R	R
B63nlf	*P.aeruginosa*	Rat	R	R	R	R
B46nlf	*P.aeruginosa*	Rat	R	R	R	S
A9nlf	*P.aeruginosa*	Rat	R	R	R	R
B28nlf	*P.aeruginosa*	Rat	R	R	R	R
A35nlf	*P.aeruginosa*	Rat	R	R	R	S
A57nlf	*P.aeruginosa*	Rat	R	R	R	S
A58nlf	*P.aeruginosa*	Rat	R	R	R	R
B54nlf	*P.aeruginosa*	Rat	R	R	R	R
A53nlf	*P.aeruginosa*	Rat	R	R	S	R
A40nlf	*P.aeruginosa*	Rat	R	R	R	R
A12nlf	*P.aeruginosa*	Rat	R	R	R	R
A41nlf	*P.aeruginosa*	Rat	R	R	R	S
B17nlf	*P.aeruginosa*	Rat	R	R	R	S
B31nlf	*P.aeruginosa*	Rat	R	R	R	R
A36nlf	*P.aeruginosa*	Rat	R	R	R	R
A45nlf	*P.aeruginosa*	Rat	R	R	R	R
B1nlf	*P.aeruginosa*	Rat	R	R	R	R
B7nlf	*P.aeruginosa*	Rat	R	R	R	S
A10nlf	*P.aeruginosa*	Rat	R	R	R	R
A51nlf	*P.aeruginosa*	Rat	R	R	R	S
A52lf	*P.aeruginosa*	Rat	R	R	R	R
A34lf	*P.aeruginosa*	Rat	R	R	R	R
B25alf	*P.aeruginosa*	Rat	R	R	R	R
A26nlf	*Proteus mirabilis*	Rat	R	R	R	S
A21nlf	*Proteus mirabilis*	Rat	R	R	R	S
A28nlf	*Proteus mirabilis*	Rat	R	R	R	R
A5nlf	*Proteus mirabilis*	Rat	R	R	R	R
B2anlf	*Proteus mirabilis*	Rat	R	R	R	R
A16nlf	*Proteus mirabilis*	Rat	R	R	R	R
B23nlf	*Proteus mirabilis*	Rat	R	R	R	R
B64nlf	*Proteus mirabilis*	Rat	R	R	R	R
A7nlf	*Proteus mirabilis*	Rat	R	R	R	R
A22lf	*Proteus mirabilis*	Rat	R	R	R	R
B4nlf	*Proteus mirabilis*	Rat	R	S	R	R
A15nlf	*Proteus mirabilis*	Rat	R	R	R	R
A48nlf	*Proteus mirabilis*	Rat	R	R	R	R
B6nlf	*Proteus mirabilis*	Rat	R	S	R	R
Ul5	*Proteus mirabilis*	Rat	R	R	R	R
Ul3	*Proteus mirabilis*	Rat	R	R	R	R
B63nlf	*Proteus mirabilis*	Rat	R	R	R	R
B19nlf	*Proteus mirabilis*	Rat	R	R	R	R
B14lf	*Proteus mirabilis*	Rat	R	R	R	R
A17lf	*Proteus mirabilis*	Rat	R	R	R	R
A6lf	*Proteus mirabilis*	Rat	R	R	R	R
Ul4	*Proteus mirabilis*	Rat	R	R	R	R

P.aeruginosa = *Pseudomonas aeruginosa*; *Ent lugwigii* = *Enterobacter lugwigii*; ceftaz = Ceftazidime; Ceftria = ceftriaxone; amik = amikacin; Cefep = cefepime; R = resistant; S = sensitive

TABLE II: NDM-1 positive Gram negative pathogen from rats captured in poultry house in Ibadan, Nigeria

Isolate	16s RNA identity	Source	Ceftaz	ceftria	amik	cefep
A15nlf	*P mirab*	Rat	R	R	R	R
B54lf	*Ent lugwigii*	Rat	R	R	R	R
Ul4	*P mirab*	Rat	R	R	R	R

P mirab = *Proteus mirabilis*; *Ent lugwigii* = *Enterobacter lugwigii*; ceftaz = Ceftazidime; Ceftria = ceftriaxone; amik = amikacin; Cefep = cefepime; R = resistant; S = sensitive

FIGURE 1: Gel picture of NDM-1 positive Gram negative bacteria from rats captured from poultry houses in Ibadan, Nigeria

Legend for FIGURE 1: Lane 1= DNA ladder, lane 19 = GenScript^{R} positive control, lanes 20 and 24= A15nlf and B54lf respectively.

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCGCT
GCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCAGCA
AATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCACCGAA
TGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCGCTTCCAA
CGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGCCTGGACCGA
TGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCG
CGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTG
CATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAA
GAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGA
ACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCA
CACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTG
CCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTG
AGCACTACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATG
ATCGTGATGAGCCATTCCGCCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGC
ATGGCCGACAAGCTGCGCTGA

FIGURE 2: Oligosequence of the positive control synthesized by Genscript

(*Proteus mirabilis*) at lane 20; B54lf (*Enterobacter lugwigii*) at lane 24 and the Genscript^{R} positive control at lane 19. Figure 2 shows the oligosequence of the synthesized positive control by Genscript^{R}.

The *bla* NDM-1 among other metallo beta lactamases (MBLs) as resistance determinants has emerged and rapidly spreading among gram negative pathogens worldwide, with great public health concerns (Bebrone, 2007;Walsh, 2010; Mulveyet al., 2011). In order to effectively control possible grievous infections by these pathogens and its spread among other pathogens, the need for continuous monitoring and rapid detection of the possible sources of the NDM bearing strains have been acknowledged (Farzana et al., 2013). For such monitoring to yield desired results, it is important to include all the possible sources of transmission of drug resistant pathogens within the food chain into consideration. We therefore screened some multidrug resistant gram negative bacteria from rats captured from poultry houses in Ibadan Nigeria for the presence of *bla*NDM-1 bearing organisms.

From our results, 5.4% (3/56) of the isolates including; 2 *Proteus mirabilis* and 1 *Enterobacter lugwigii* were positive for the

gene with the expected band of about 814bp. This small percentage notwithstanding, the detection of the gene among bacteria carried by rats in association with poultry poses a public health threat, because the rats can serve sources for disseminating the public health important genes through these bacteria to other poultry and human pathogens through the food chain thus constituting great public health threat.

Conclusion: The result from this work showed the need for public health education/enlightenment on the possible risk associated with the involvement of bacteria isolated from rats in spreading the gene to other animal and human pathogens. In lieu of these findings, rats control in the animal houses and households become indispensable.

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