Research Article

Detection of Quinolone Resistance through Amplification of the gyrA Gene of *Mycobacterium* species from Human and Animal Sources

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ABSTRACT

Ten (10) DNA samples of *Mycobacterium tuberculosis* (*Mtb*) isolated from sputum of TB-positive humans, DNA samples from Mycobacterium species isolated from lymph nodes and fecal samples of avians and bubaline animals were analysed by PCR targeting primers for gyrase A (gyrA), quinolone resitance A(qnrA) and topoisomerase IV (parC) genes. Results demonstrated that quinolone resistance recognized by gyrA was seen in one out of 10 DNA samples from human *Mtb* isolates and that no qnrA and parC genes were detected. The gene for quinolone resistance detected by the primer gyrA had a molecular weight of 333 bp. Resistance to quinolone mediated by gyrA, qnrA and parC genes in avian (*M. avium avium*) and bubaline (*M. avium paratuberculosis*) isolates of mycobacteria were not detected after PCR. The non-amplification of genes observed in this study explains the non-existence of quinolone resistance arbitrated by gyrA, qnrA and parC genes in the specified avian and bubaline mycobacterial isolates.

Key words: *Mycobacterium avium avium, Mycobacterium avium paratuberculosis, Mycobacterium tuberculosis*, Polymerase chain reaction, Quinolone resistance

INTRODUCTION

Fluroquinolones are synthetic antibiotics that inhibit nucleic acid synthesis in bacteria. Original preparations of quinolones reportedly act against enteric bacteria and some facultative Gram-negative microbes. The fluorinated quinolones, now known as fluoroquinolones, were purportedly made from original preparations to augment the spectrum of activity against other bacteria like *Pseudomonas*, Gram-positive and Gram-negative bacteria. Reduced sensitivity of bacteria to the original classes of quinolones reportedly observed by many researchers parallel build up of resistance to the drug (Fung-Tome *et al.,* 1993).

Currently available preparations of fluoroquinolones used in human medicine include moxifloxacin, gatifloxacin, levofloxacin, ofloxacin and ciprofloxacin (Ginsburg *et al.,* 2003; Huang and Stafford, 2002; Wallace *et al.,* 1990). In veterinary practice, fluoroquinolones such as enrofloxacin, levofloxacin and ciprofloxacin are used as vital medications for many systemic infections of livestock, poultry and fish in many parts of the world. The use of these drugs in livestock presents an area of concern due to the possible transmission of antimicrobial resistance to humans via the food chain (Endtz *et al.,* 1991). Bacteria that develop resistance to quinolones generally develop cross-resistance to other quinolones and this cross-resistance reportedly covers quinolones used in animals and humans. Bacterial exposure to lower concentrations of quinolones increases build-up of resistance (Hooper, 2011). Inadequate dosing of quinolones, different routes of administration and treatment of water and feed supplementation with quinolones are common controversies that underlie induction of resistance (Herikstad *et al.,* 1997). With the introduction of advanced methods in molecular biology, various mechanisms such as chromosomal mutation (Takiff*et al.,* 1994), plasmid-intervention (Tran *et al.,* 2009) and alteration of DNA gyrase (Cambau *et al.,* 1994) have been identified in the acquisition of quinoloneresistance by bacteria.

Literature that describes the status of quinolone resistance in the medical field is quite extensive while it is limited in veterinary practice. Massive differences in resistance patterns of bacterial pathogens to quinolones may occur across laboratories, veterinarians, pharmaceutical companies and food-producing agencies. A research-generated data detected the existence of quinolone resistance in *Mycobacterium* isolates of animal and humans. This does not intend to threaten the usefulness of the drug as a treatment for medical and many veterinary important infections but this has to be taken as leverage in the rational selection of antimicrobials for therapeutic purposes.

MATERIALS AND METHODS

Mycobacterium samples from animal and human sources for evaluation

Fecal samples (50 gms) and lymph nodes from 5 out of 30 Philippine water buffaloes and one (1) out of 30 gamecocks that tested positive to TB testing were collected for the study. Water buffaloes that were positive to TB testing had skin bleb that measured 21.0 to 22.95 mm diameter, while chickens that were positive to the test had 24.45 mm skin bleb diameter 48 hrs- post test (Soriano, 2016). Samples collected from water buffaloes and chickens were inoculated in Middle Brook medium supplemented with glycerol and mycobactin. Samples were incubated at 37ºC for 21 to 28 days. *Mycobacterium tuberculosis (Mtb)* samples were cultivated in Lowenstein medium undertaken under biosafety laboratory conditions at the Tropical Disease Foundation, Makati City, Philippines. The isolates from water buffaloes and from game cocks were respectively identified as *Mycobacterium avium paratuberculosis (Map)* and *Mycobacterium avium avium (Maa)* based on their DNA sequences (Palma, 2016).

Extraction of Genomic DNA

Samples of *Map* from water buffaloes and *Maa* from chickens cultivated in Middle Brook and *Mtb* from Lowenstein medium were transferred aseptically in separate tubes. Samples were heated at 95°C for one min which was repeated 10 times before the addition of 500 µL lytic enzyme. The samples were incubated at 37°C for 60 min and then centrifuged for 2 min at 13 000 x g. The protocol for DNA extraction described by the manufacturer (Wizard Genomic DNA Purification kit, Promega, USA) was followed. The supernatants were removed before resuspending the cells in 600 µL nuclei lysing buffer. Samples were separately incubated at 80°C for 5 min, allowed to cool at room temperature before the addition of RNase solution (3 µL). Samples were incubated at 37°C for 60 min and allowed to cool at room temperature before adding the protein precipitation solution (200 µL). Samples were incubated on ice for 5 min then centrifuged at 13 000 x g for 3 min. Each supernatant sample was transferred to clean microcentrifuge tubes that contained 600 µL isopropanol, mixed and centrifuged at 13 000 x g for 2 min. Sample supernatants were collected in tubes for the DNA samples to dry before the addition of 600 µL

ethanol (70%). Samples were again centrifuged at 13 000 x g for 2 min before removal of the ethanol. The samples were allowed to dry. DNA rehydration solution (100 µL) was added to each sample then incubated at 65°C for 1 hr before PCR evaluation.

Amplification of quinolone resistance genes in Mycobacterium samples from animal and human sources

The primers for gyrase A (gyrA), quinolone resitance A (qnrA) and topoisomerase IV (parC) genes were used as described by Cano *et al.* (2009). PCR methods were optimized for each gene utilizing the 3primers that recognize gyrA enzyme-related alterations; parC mutation; and plasmid-mediated quinolone resistance (Table 1). The reaction mixture for PCR consisted of 4.45 µL DDW, 2 µL 5X PCR buffer (Promega, USA), 1 µL MgCl$_2$(Promega, USA), 0.5 µL dNTP (Intron, Korea), 0.5 µL forward primer (Integrated DNA Technologies), 0.5 µL reverse primer (Integrated DNA Technologies), 0.05 µL Taq polymerase (Promega, USA) and DNA template (1 µL) making up a total volume of 10 µL. Thermal cycling involved initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, 1 min extension at 72°C, a final extension at 72°C for 10 min and a final hold at 4°C (ThermoFisher Scientific, USA). The PCR products and the bp ladder (3 µL) were separately loaded in wells of 2% agarose gel with gel red nucleic acid stain (Biotium Inc., USA) and after electrophoresis amplified products were evaluated in a trans-illuminator (GelDoc, USA).

DNA sequencing of quinolone resistance genes

Two (2) samples of the amplified products were used for sequencing. The gene sequence for quinolone resistance was aligned using BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Center (NCBI).

RESULTS AND DISCUSSION

Detection of quinolone resistance in Mtb from human sources

Data show that one of the 10 *Mtb* DNA samples registered a gene with quinolone resistance and this was recognized by the pair of gyrA primers (Fig. 1). The gene had a molecular weight of 340 bp. Neither parC nor qnrA genes were detected.

This datum validates the presence of quinolone resistance in *Mtb* and it is noteworthy that the observed resistance may be associated with an alteration of the gene that regulates the bacterial enzyme gyrA. Results point out the detection of quinolone resistance in a few number (1 out of 10) of human isolates of *Mtb* contrary to a previous report on high rates of resistance to quinolone preparations such as ciprofloxacin and ofloxacin among TB patients in tertiary hospitals in the Philippines (Grimaldo *et al.*, 2001). This information is quite alarming because of the reported resistance to quinolone-based anti-tuberculosis therapeutic agents.

Table 1: Primers used in the detection of quinolone resistance of *Mycobacterium* samples from animal and human sources

Primers	Sequence	Product Length (bp)	Target	Author	Accession Number
gyrA	F 5'-AAA TCT GCC CGT GTC GTT GGT-3'	343	Gyrase A	Vila *et al.*, 1995	EMBL X82165
	R 5'-GCC ATA CCT ACG GCG ATA CC-3'				
parC	F 5'-ATG TAC GTG ATC ATG GAC CG-3'	300	Mutation	Cano *et al.*, 2009	GenBank (KJ756512, KJ 756513 and KJ756514)
	R 5'-ATT CGG TGT AAC GCA TCG CC-3'				
qnrA	F 5'-GAT AAA GTT TTT CAG CAA GAG G-3'	543	Plasmid	Jacoby *et al.*, 2003	GenBankAY070235
	R 5'-ATC CAG ATC GGC AAA GGT TA-3'				
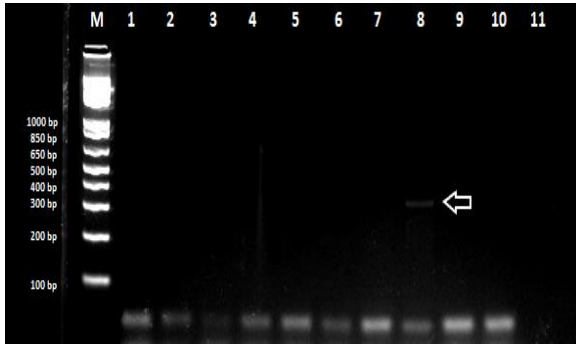
Fig. 1: Amplification of quinolone resistance gene in *Mtb* samples from humans recognized by the gyrA primers. M (1 kb + molecular ladder); Lanes 1 to 10 (10 *Mtb* DNA samples); Lane 8, positive to gyrA gene; Lane 11 (Negative control).
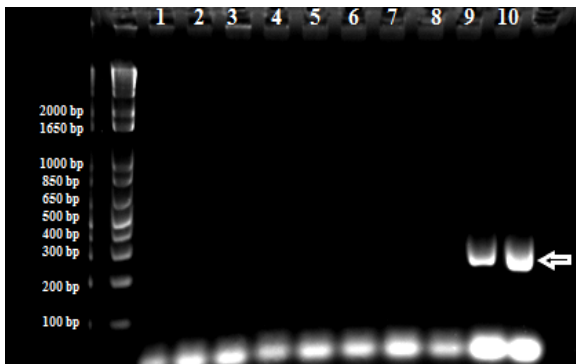
Fig. 2: Non-amplification of quinolone resistance gene in *Mycobacterium* samples from animal sources using gyrA primers. M (1 kb plus ladder); Lanes 1 to 3 (*Maa* isolates), Lanes 4 to 6 (*Map* isolates), Lanes 7 and 8 (Sterile distilled water, Negative control); and Lanes 9 and 10 (*Mtb* isolate, Positive control).
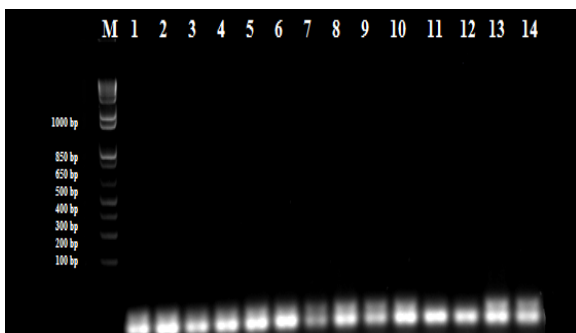
Fig. 3: Non-amplification of quinolone resistance gene in *Mycobacterium* samples from animal sources using parC (Lanes 1 to 7) and qnrA primers (Lanes 8 to 14). M (1 kb + ladder); Lanes 1 to 3 (*Maa* isolates), Lanes 4 to 6 (*Map* isolates), Lane 7 (Sterile distilled water, Negative control for parC primer evaluation); Lanes 8 to 10 (*Maa* isolates), Lanes 11 to 13 (*Map* isolates) and Lane 14 (Sterile distilled water, Negative control for qnrA primer evaluation).

Detection of quinolone resistance in *Maa* and *Map* from animal sources

Investigation of quinolone resistance in *Mycobacterium* samples from animal sources also applied the same sets of primers for gyrA, parC and qnrA. Results showed no PCR amplification for gyrA gene in animals (Fig. 2). Results of supplementary experiments utilized primers for parC and qnrA genes for the evaluation of quinolone resistance in *Mycobacterium* samples from animal sources. Data did not demonstrate amplification of the parC and qnrA genes (Fig. 3).

The present study demonstrates that quinolone resistance mediated by gyrA, parC and qnrA genes were not observed in *Mycobacterium* spp. obtained from water buffaloes and game cocks. This, however, does not rule out the idea that resistance to quinolone may take place in animal isolates of *Mycobacterium* spp. but the mechanisms may be different from those mediated by the genes gyrA, parC and qnrA. The limited samples of *Mycobacterium* spp. from TB-positive avian and bubaline animals for analysis in which quinolone resistance was not apparently observed, may imply that the antibiotic is not commonly and frequently used as a medication in these species of animals in the place of collection. This may demonstrate that non-frequent utilization of an antibiotic may not likely induce development of resistance to a drug unlike repeated administration and excessive use of antibiotics would do.

DNA sequence of the gene responsible for quinolone resistance

The aligned sequence of the quinolone-resistance gene detected by the primer gyrA is shown in Table 2. The sequence is composed of 333 nucleotides with a 98% homologous identity with the gene sequences of bacteria that carry quinolone resistance described in the GenBank. Resistance to antibiotics has evolved as a major problem in the medical field. This reportedly poses health and economic concerns as it leads to the difficulty of treatment against certain diseases and imparts losses due to administration of antibiotics which may not ensure effective treatment. It purportedly escalates production costs due to the repeated use of antibiotics which do not ensure recovery of animals from existing diseases (Acar, 1997).

Quinolones are described as bactericidal broad-spectrum antibiotics effective against many different types of bacteria, both Gram-positive and Gram-negative (Oliphant and Green, 2002). Quinolones and its derivatives are the most promising anti-tuberculous therapeutic agents and are widely used for the treatment of multidrug-resistant tuberculosis (Eldin *et al.*, 2012).

Table 2: DNA sequence of quinolone resistance gene recognized by gyrA

Sequence	Product length(bp)
AAATCTGCCCGTGTCGTTGGTGACGTAATCGGTAAATACCATCCCCATGGTGATTCCGCGGTGTACGA CACTATCGTCCGTATGGCGCAGCCTTTCTCGCTGCGTTACATGCTGGTGGATGGCCAGGGTAACTTTG GTTCTATCGACGGCGACTCCGCCGCGGCAATGCGTTATACGGAAATCCGTCTGGCGAAAATTGCCCAT GAGCTGATGGCCGACCTGGAAAAAGAGACGGTTGATTTCGTTGATAACTACGACGGCACGGAAAAAA TTCCTGACGTCATGCCAACGAAATACCTAATCTGCTGGTAAACGGTTCGTCCGGTATCGCC	333

The application of the primers for gyrA, parC and qnrA has been noted as efficient protocol for determining quinolone resistance in *Mycobacterium* isolates from human and animals. Few studies have explored quinolone resistance in bacteria targeting the gyrA gene. The group of Eldin (2012) determined fluoroquinolone resistance in *Mtb* with the use of gyrA and B primers. Primers for the DNA gyrase gene were also applied by Dauendorffer et al. (2003) in the evaluation of quinolone-resistance-determining-regions (QRDR) of *Mycobacterium* spp.

The quinolone resistance detected in this study follows the findings of others (Hooper, 2011) who related fluoroquinolone resistance to alteration of DNA gyrase and topoisomerase IV. The modification of DNA gyrase function resulting to antimicrobial resistance has been described to imply derangement of an active site for double stranded DNA breakage and reunion, control of DNA recognition and a catalyst for supercoiling of DNA (Oliphant and Green, 2002). Another mechanism of resistance to quinolone reportedly involves mutations that alter drug targets such as the bacterial enzymes DNA gyrase and DNA topoisomerase (Drlica and Zhao, 1997). Mutation-mediated resistance reportedly involves amino acid substitution in the DNA binding region of the gyrA or parC subunits referred to by scientists as QRDR (Cabral *et al.,* 1997) and as "hot spots" for mutation in the DNA gyrase of *E. coli* (Friedman *et al.,* 2001). Barnard and Maxwell (2001) provided a thorough discussion that once initial mutation has taken place and reduced the susceptibility of DNA gyrase to quinolone, additional mutation in gyrA gene augments resistance and renders quinolone ineffective against a bacterium. Other scientists report that mutation impairs target enzyme function, reduces formation of enzyme-DNA complexes that bind on quinolone and arrests the enzyme-mediated break in double-stranded DNA and the sealing of the break with a second double helix DNA (Ince and Hooper, 2003).

Future efforts that involve analysis of gene components will have to be done to understand the mechanism of resistance of animal-borne *Mycobacterium* spp. to quinolones. It is through research that the complexity behind antimicrobial resistance is better understood so that avenues intended to counter this problem and attain success in antimicrobial therapy can be achieved.

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