Review Article

Characterization of β-Lactam-Resistant *Escherichia coli* Strains Isolated From Catfish (*Ictalurus punctatus*) Raised without the Drug

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Abstract

Fifty-two *Escherichia coli* strains were isolated from farm-raised catfish (*Ictalurus punctatus*). All isolates were resistant to ampicillin and penicillin. The Minimum Inhibitory Concentrations (MIC) of both drugs for these isolates were between 64-256 μg/mL. Template DNA of the isolates was screened for the presence of 11 different β-lactam resistance genes. Oligonucleotide primers specific for the detection of bla_{TEM} amplified the gene in 41/52 (79.0%) of the isolates; primers for bla_{CTX-M} amplified the gene in 28/52 (54.0%) of the isolates; and primers for bla_{SHV} detected the gene in 3/52 (5.0%) of the isolates. PCR-Based Replicon Typing (PBRT) was used for plasmid identification and for targeting the replicons of the 15 major plasmid families in the template DNA of the isolates. Primers specific for the incompatibility group (Inc)- FIB plasmid successfully amplified a 702-bp region of the replicon from the template DNA of 36/52 (69.0%) of the isolates; primers for a 159-bp region of the Inc-B/O plasmid replicon detected the plasmid from the template DNA of 33/52 (63.0%) of the isolates; and primers for a 462-bp region of the Inc-FIA plasmid replicon detected the plasmid in 18/52 (35.0%) of the isolates. Efflux pump activity was determined by ethidium bromide accumulation and measurement of Relative Fluorescence Units (RFU). Efflux pump activity was higher in multidrug resistant isolates relative to antibiotic-susceptible strains of *E. coli*. The β-lactam resistance determinants, along with the plasmid replicons Inc-FIB and B/O, were successfully transferred to the sodium azide-resistant *E. coli* J53 recipient. Our results indicate that the catfish ecosystem is a reservoir of β-lactam resistance.

Keywords: Beta-lactam Resistance; Catfish; *E. coli*

Introduction

Pond-raised catfish (*Ictalurus punctatus*) is the largest aquaculture product in the United States and is mainly cultured in Arkansas, Alabama, Mississippi and Louisiana. These four Southern states together produce more than $350 million of pond-raised catfish annually [1]. Intensive catfish production can be affected by numerous infectious diseases that can severely curtail production [2]. Several major classes of antimicrobials are being used, or have been used, in global aquaculture practices [3-5]. Noteworthy among these antimicrobials are penicillins, macrolides, quinolones, phenicols and tetracyclines. However, only oxytetracycline and Romet 30 (sulfadimethoxine-ormetoprim) are currently approved by the US FDA (Posadas 2017). Reports have characterized the occurrence and prevalence of antibiotic resistance determinants to these drugs [6,7]. However, very little is known about antibiotic resistance mechanisms to drugs not approved specifically for usage in catfish aquaculture in the US, although the antibiotic resistance determinants to these drugs in catfish may be transferred to bacteria in clinical and other ecosystems [6,8].
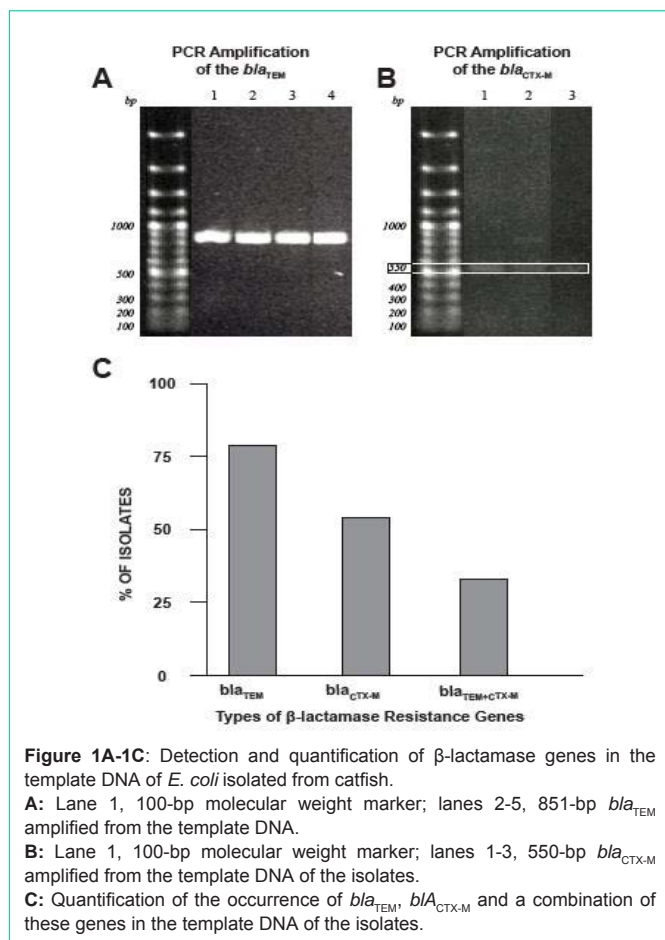
β-lactam antibiotics (including penicillin derivatives, cephalosporins, monobactams and carbapenems) are widely used in clinical practices for the treatment of numerous human ailments [9]. The intense use of these drugs in aquaculture ponds to prevent the outbreak of diseases may select bacteria resistant to these life saving drugs and may contribute to reduced efficacy of these drugs in the treatment of infectious clinical diseases [8]. Currently, several mechanisms of resistance to β-lactam drugs have been reported [10,11]. Additionally, several other mechanisms, such as altered cell membrane permeability and overexpression of multidrug efflux pumps, also contribute to drug resistance [12]. However, very little is known on the prevalence of various β-lactam resistance determinants and the replicon types of plasmid that harbor β-lactam resistant genes in catfish ecosystems. Such information will be crucial in understanding the epidemiology of the prevalence of these determinants and will aid in devising mitigating strategies to curtail the spread of β-lactam resistance. In this report, we describe the antibiotic resistance profiles and the molecular characterization of β-lactam resistance in 52 *E. coli* strains isolated from catfish.

Materials and Methods

Isolation, characterization and identification

Bacteria were isolated from the intestines of catfish collected from 16 commercial ponds in Arkansas, Louisiana and Texas [7]. One gram of each sample was enriched for 6 h in Luria Broth (LB). Enriched samples were streaked on MacConkey agar plates and

Figure 1A-1C: Detection and quantification of β-lactamase genes in the template DNA of *E. coli* isolated from catfish.

A: Lane 1, 100-bp molecular weight marker; lanes 2-5, 851-bp *bla*TEM amplified from the template DNA.

B: Lane 1, 100-bp molecular weight marker; lanes 1-3, 550-bp *bla*CTX-M amplified from the template DNA of the isolates.

C: Quantification of the occurrence of *bla*TEM, *blA*CTX-M and a combination of these genes in the template DNA of the isolates.

incubated at 37°C overnight. Presumptive positive colonies of *E. coli* were biochemically characterized and identified by the Vitek GNI+ card with VTK-R07-01 software (bioMerieux Vitek, Hazelwood, MO) and by fatty acid methyl ester analysis (MIDI, Newark, DE). All isolates were stored in LB containing 20% glycerol at -70°C and were grown overnight at 37°C in LB broth or on Trypticase Soy Agar (TSA) plates supplemented with 5% sheep's blood.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to β-lactams of each *E. coli* isolate was determined by the disk diffusion method [13] using the criteria specified by the Clinical and Laboratory Standards Institute [14].

Genomic DNA Extraction

The genomic DNA was isolated from bacterial cells grown overnight at 37°C using the QIAamp DNA mini kit (QIAGEN, Valencia, CA).

Detection of β-Lactamase Genes from Template DNA

The presence of various β-lactam resistance genes in the template DNA was determined by PCR [15]. The primers used for the amplification of these genes are listed in Table 1. PCR amplification of the β-lactam resistance genes was carried out in a reaction volume of 25 μL. The thermal cycling conditions consisted of an initial denaturation of 94°C for 2 min followed by 35 cycles of amplification. Each cycle consisted of 94°C denaturation for 30 s, annealing for 1°C

below the lowest Tm of a given primer pair, and 72°C extension for 1 min. The amplified PCR products were maintained at 4°C. A reagent blank contained all the components of the reaction mixture except template DNA, for which sterile distilled water was substituted. The PCR products were subjected to electrophoresis on 1.2% agarose gels in 1 mM Tris-borate-EDTA (TBE) buffer, visualized with UV, and photographed using an Eagle Eye II gel documentation system (Stratagene, La Jolla, CA). A 100-bp DNA ladder (Thermo Fisher Scientific, Grand Island, NY) was used as the size standard.

Plasmid Typing

Plasmids were typed by the PCR-Based Replicon Typing (PBRT) method [15,16] with primers listed in Table 2.

Estimation of Efflux Pump Activity

Accumulation of Ethidium Bromide (EtBr) was monitored as described elsewhere [17]. Fluorescence was read on a Synergy 2 Multiple-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO), an efflux pump inhibitor, was added to the assay mixture at a final concentration of 100 μM. The natural fluorescence of the cells was subtracted and the fluorescence intensity was expressed in Relative Fluorescence Units (RFUs). All experiments were performed in triplicate.

Conjugation

Four ampicillin-resistant, but sodium azide-sensitive *E. coli* strains (ECT 660, ECT 330, ECT 10 and ECT 711) isolated from catfish samples were selected as donors. A 256-μg ampicillin-sensitive but 800 μg/mL sodium azide-resistant *E. coli* J53 strain was used as a recipient for conjugation experiments to determine transferability of β-lactamase resistance genes. The broth mating conjugation method was performed as previously described [18]. LB agar plates containing ampicillin (256 μg/ml) and sodium azide (800 μg/mL) were used to select transconjugants harboring genes conferring β-lactam and sodium azide resistance.
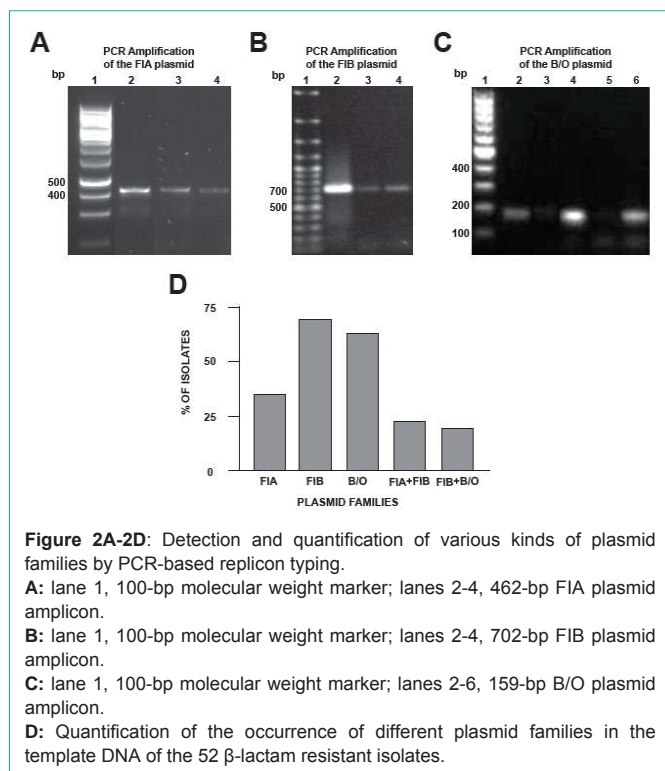
Results

Beta-lactam Resistance Profiles of *E. coli* strains Isolated from catfish

All 52 isolates were resistant to ampicillin and penicillin. For four isolates (ca. 8%), the MIC values of ampicillin and penicillin were 64 μg/mL, for 38 isolates (73%), they were 128 μg/mL and for 10 isolates (19%) they were 256 μg/mL. Sixteen isolates (ca. 31%) were resistant to cephalothin (30 μg) and ampicillin (30 μg). Six isolates (12%) were resistant to ampicillin, ceftriaxone, ceftazidime and amikacin. Five isolates (10%) were resistant to ceftazidime, ceftriaxone, cephalothin, amikacin and ampicillin. Another five isolates (10%) were resistant to amikacin, ampicillin, ceftazidime, ceftriaxone and cephalothin.

PCR Amplification of β-lactam Resistance Genes

The template DNA of the 52 β-lactam antibiotic-resistant strains was screened for the presence of 11 different β-lactam resistance genes. Oligonucleotide primers specific for the amplification of the ESBL gene *bla*TEM amplified the 851-bp region of this gene from the template DNA of 41 of the 52 *E. coli* strains (79%) (Figure 1A). Primers specific for the amplification of *bla*CTX-M amplified a 550-bp region of the gene from the template DNA of 28 of the isolates (54%)

Figure 2A-2D: Detection and quantification of various kinds of plasmid families by PCR-based replicon typing.
A: lane 1, 100-bp molecular weight marker; lanes 2-4, 462-bp FIA plasmid amplicon.
B: lane 1, 100-bp molecular weight marker; lanes 2-4, 702-bp FIB plasmid amplicon.
C: lane 1, 100-bp molecular weight marker; lanes 2-6, 159-bp B/O plasmid amplicon.
D: Quantification of the occurrence of different plasmid families in the template DNA of the 52 β-lactam resistant isolates.

Figure 3: Quantification of the efflux pump activity in the β-lactam resistant *E. coli* strains isolated from catfish. Relative fluorescence units (RFUs) were determined in a Synergy 2 multiple mode microplate reader. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), an active efflux pump inhibitor, was used in the assay. RFU values of bacterial cells resistant to six different antibiotics treated with CCCP at 0 min (▲), RFU of bacterial cells resistant to six different antibiotics treated with CCCP after 30 min incubation (△), RFU of bacterial cells resistant to four different antibiotics treated with CCCP at 0 min (■) and after 30 min incubation (□).
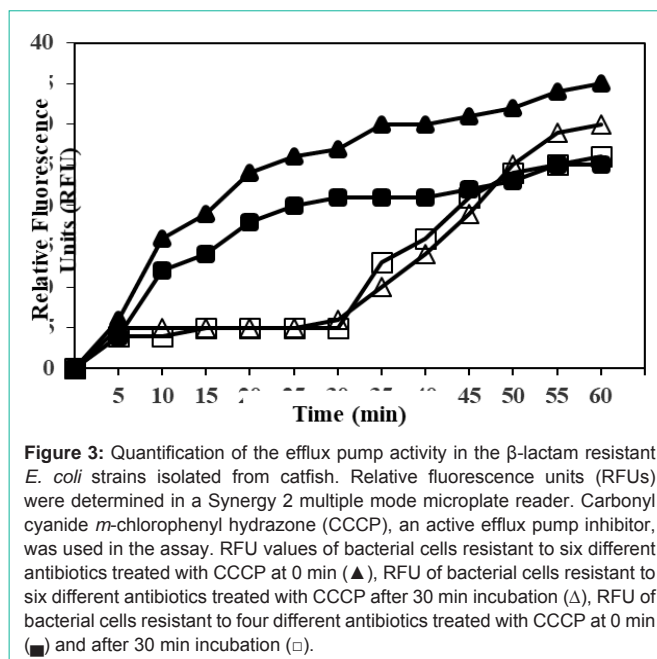
(Figure 1B). PCR also detected the presence of bla_{SHV} in three of the 52 (5%) isolates (data not shown). The template DNA of 22 isolates (42%) harbored both bla_{TEM} and bla_{CTX-M} genes (Figure 1C), and that of three isolates harbored all three β-lactam genes (bla_{TEM}, bla_{CTX-M} and bla_{SHV}, data not shown). PCR failed to amplify the other eight β-lactam resistance genes from the template DNA of any of the 52 isolates.

Plasmid Identification and Typing

PCR-Based Replicon Typing (PBRT) was used for plasmid identification, targeting the replicons of 15 major plasmid families in the template DNA of the 52 β-lactam-resistant strains of *E. coli*. Primers specific for the identification of the Inc-FIA plasmid successfully amplified a 462-bp region of the replicon from the template DNA of 18 of the 52 isolates (35%) (Figure 2A-2D). Primers specific for the Inc-FIB plasmid successfully amplified a 702-bp region of the replicon from the template DNA of 36 of the 52 isolates (69%) (Figure 2B-2D). Similarly, primers specific for a 159-bp region of the Inc-B/O plasmid replicon amplified a part of the plasmid from the template DNA of 33 of the 52 isolates (63%) (Figure 2C-2D). PBRT indicated that 12 of the 52 isolates (23%) harbored both Inc-FIA and Inc-FIB plasmids (Figure 2D) and 19% of the isolates contained both Inc-FIB and Inc-B/O plasmid replicons. The primers failed to amplify any of the other 12-plasmid families from the template DNA of the isolates.

Ethidium Bromide Accumulation Assay

Accumulation of EtBr in β-lactam-resistant strains and sensitive control strains of *E. coli* was tested in the presence and absence of the efflux pump inhibitor CCCP and measured by Relative Fluorescence Units (RFU). The accumulation of EtBr increased gradually with incubation time, up to 40 min after treatment, in all β-lactam-resistant

E. coli strains treated with EtBr (Figure 3). More accumulation of EtBr at 40 min was observed in the isolates resistant to six antibiotics than in the isolates resistant to only four drugs. The maximum RFU, indicating EtBr accumulation, in *E. coli* isolates resistant to six antibiotics was ca. 35 and the RFU in isolates resistant to only four antibiotics was ca. 25. Higher RFU values were obtained when the efflux pumps were blocked by CCCP after 30 min incubation. RFU values peaked at ca. 33-35 in isolates resistant to six antibiotics but at 23-25 in isolates resistant to only four drugs. Efflux pump activity was also observed in antibiotic-susceptible control strains of *E. coli*. However, the efflux pump activities of these strains were inferior (RFU 10-12) to those of the antibiotic-resistant strains.

Horizontal Transfer of β-lactam Resistance Phenotypes and Genotypes

Four strains (*E. coli* strains ECT 660, 330, 10 and 711) that were resistant to 256 μg/mL of ampicillin but sensitive to 800 μg/mL of sodium azide were selected as donors. Our preliminary analysis indicated that these strains harbored bla_{TEM} and bla_{CTX-M} and carried FIB and B/O plasmids. Transconjugants were readily obtained with all four mating donor strains and strain J53. All transconjugants were resistant to 256 μg/mL of ampicillin and 800 μg/mL of sodium azide. The template DNA from all transconjugants was positive for the presence of the 851-bp bla_{TEM}, 550-bp bla_{CTX-M}, 702-bp FIB plasmid and 159-bp B/O plasmid replicons. Serial dilution techniques indicated the rates of transconjugation to be 6.1×10^4 and 3.9×10^4 with ECT 660 and ECT 10, respectively, but only 7.2×10^3 and 5.1×10^3 when strains 330 and 711, respectively, were used as donors.

Discussion

Extended Spectrum β-Lactamases (ESBL) are often located on plasmids and play a crucial role in conferring broad resistance to β-lactam antibiotics [11,19-21]. Currently, more than 300 ESBL variants have been described in Gram-negative bacteria; most are derivatives of TEM or SHV enzymes [11]. The most common group

of ESBLs not belonging to the TEM or SHV families is termed CTX-M and has greater activity against newer generations of β-lactams [11,22]. ESBLs hydrolyze and inactivate a wide variety of β-lactams, including third-generation cephalosporins, penicillins, ampicillins and aztreonams (Canton et al. 2012) [22]. Several investigations have indicated that these three β-lactam resistance determinants are widely prevalent in most ESBL-resistant *E. coli* and *K. pneumoniae* strains [11,19-21]. These determinants are also found in other genera of Enterobacteriaceae [11,22-23].

Numerous reports have indicated the widespread occurrence of β-lactam resistance determinants, including ESBLs, in *E. coli* strains isolated from food products, healthy humans, and livestock, as well as in clinical isolates [19-21,24]. Little or no information is available on the occurrence and prevalence of β-lactam resistance determinants in catfish aquaculture in the [25] investigated the distribution of various β-lactam resistance determinants from eels and aquaculture ponds. These investigators concluded that bla_{TEM} was found in the template DNA of all 108 strains of antibiotic-resistant bacteria isolated from aquaculture ponds, followed by bla_{SHV} (15.0%). Another report indicated that bla_{TEM} was detected in 29 of 30 strains of *E. coli* (97.0%) isolated from seafood [26]; no other β-lactam resistance determinants were detected in this study.[5] reported that bla_{TEM} was the dominant β-lactam resistance gene in 100% of all strains isolated from urban aquatic environments in India, followed by bla_{CTX-M} in 16.0% of the isolates. Molecular screening for 11 different β-lactam resistance genes in the template DNA of 52 multiple antibiotic-resistant isolates in our study indicates a variety of β-lactam resistance determinants. Many of the isolates harbored more than one β-lactam resistance determinant. Our results concur with the widespread distribution of β-lactam resistance genes in aquatic environments.

We correlated the MIC values for penicillin and ampicillin of each isolate and the prevalence of β-lactam resistance determinants in these isolates. Our data indicate that 30 isolates that had MIC values for penicillin and ampicillin of 128 μg/mL harbored both bla_{TEM} and bla_{CTX-M} genes, but four isolates that had an MIC of 64 μg/mL harbored only one β-lactam resistance gene. It is possible that simultaneous occurrence of two or more ESBL genes may be necessary for higher MIC values in these isolates.

The extensive prevalence of ESBLs is often associated due to the horizontal gene transfer via plasmids [12,27-29]. Several investigations have reported that the replicon types most frequently detected in ESBLs among Enterobacteriaceae belong to the incompatibility (Inc) group, which includes F, A/C, L/M, I1, H12 and N. IncF and IncI1 are the most frequently reported replicon types associated with the dissemination of ESBLs. ESBL bla_{CTX-M} has been found on an IncF plasmid belonging to type FII in combination with FIA [30,31]. These plasmids with different replicon types have been identified in strains isolated from different ecosystems (environment, livestock and humans). Contrary to these investigation, none of the isolates in our investigation were found to harbor any of the replicon types Inc-F, A/C, L/M, I1 or H12. We for the first time report the widespread prevalence of replicon types FIB and B/O in *E. coli* isolated from catfish. Our investigations also found the less frequent occurrence (35.0%) of the replicon type FIA in *E. coli* in catfish ecosystems. Our results indicate that aquaculture may be a reservoir for these replicon types, which may play a vital role in the dissemination of ESBL determinants [32].

The β-lactamase genes may not be the sole contributing factor for bacterial resistance to these antibiotics and reports have indicated the involvement of other possible mechanisms in conferring resistance to antibiotics (Bush 2010). Efflux pumps are known to play a significant role in decreased accumulation of antibiotics and increased resistance to these drugs [33,34]. Our data indicate the active presence of efflux pumps in all β-lactam-resistant *E. coli* strains isolated from catfish. Thus, the high level of β-lactam resistance in *E. coli* isolated from catfish may be due to a combination of various β-lactam resistance genes plus active efflux pumps.

Although β-lactam antibiotics are not approved for use in catfish aquaculture in the U.S., the prevalence of various β-lactam resistance determinants in *E. coli* isolated from catfish indicates that catfish aquaculture may be a reservoir of β-lactam resistance determinants. Since the β-lactam resistance determinants are plasmid-borne, the *E. coli* strains in catfish may have acquired the β-lactam resistance determinants from other ecosystems [24,35-37]. Therefore, knowledge regarding the prevalence of such determinants is of paramount importance in developing effective methods to mitigate widespread antibiotic resistance.

Disclaimer

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