Development of Quinoxaline 1, 4-Dioxides Resistance in Escherichia coli and Molecular Change under Resistance Selection

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Abstract

Quinoxaline 1, 4-dioxides (QdNOs) has been used in animals as antimicrobial agents and growth promoters for decades. However, the resistance to QdNOs in pathogenic bacteria raises worldwide concern but it is barely known. To explore the molecular mechanism involved in development of QdNOs resistance in Escherichia coli, 6 strains selected by QdNOs in vitro and 21 strains isolated from QdNOs-used swine farm were subjected to MIC determination and PCR amplification of oqxA gene. A conjugative transfer was carried out to evaluate the transfer risk of QdNOs resistant determinant. Furthermore, the transcriptional profile of a QdNOs-resistant E. coli (79O4-2) selected in vitro with its parent strain 79–161 was assayed with a prokaryotic suppression subtractive hybridization (SSH) PCR cDNA subtraction. The result showed that more than 95% (20/21) clinical isolates were oqxA positive, while all the 6 induced QdNOs-resistant strains carried no oqxA gene and exhibited low frequency of conjugation. 44 fragments were identified by SSH PCR subtraction in the QdNOs-resistant strain 79O4-2. 18 cDNAs were involved in biosynthesis of Fe-S cluster (narH), protein (rpoA, trmD, truA, glyS, ileS, rplFCX, rpsh, fusA), lipote (lipA), lipid A (lpxc), trehalose (astA), CTP(pyrG) and others molecular. The 11 cDNAs were related to metabolism or degradation of glycolysis (gpmA and pgi) and proteins (clpx, clpa, pepN and fbpB). The atpADG and ubiB genes were associated with ATP biosynthesis and electron transport chain. The pathway of the functional genes revealed that E. coli may adapt the stress generated by QdNOs or develop specific QdNOs-resistance by activation of antioxidative agents biosynthesis (lipoate and trehalose), protein biosynthesis, glycolysis and oxidative phosphorylation. This study initially reveals the possible molecular mechanism involved in the development of QdNOs-resistance in E. coli, providing with novel insights in prediction and assessment of the emergency and horizontal transfer of QdNOs-resistance in E. coli.

Introduction

Quinoxaline-1,4-dioxides (QdNOs) have been introduced into animal production as potent antibacterial agents against most gram-positive and gram-negative pathogens since 1970s [1,2,3]. These drugs have been also widely used as growth promoters for decades [4,5]. Carbadox, maquindox and olaquindox are the well known members of QdNOs [6]. Cyadox is a new member of QdNOs which exhibits better antibacterial activity, lower toxicity and higher efficiency on animal growth promotion [7,8,9,10].

It is demonstrated that usage of olaquindox on pig farms may increase the emergence of olaquindox resistance in some pathogens [11,12]. The bacteria with resistance to olaquindox are generally co-resistant to ampicillin, tetracycline or chloramphenicol [1,13]. For the mechanism of QdNOs resistance, it is only known that plasmid-encoded multidrug efflux pump OqxAB confers resistance to ampicillin, chloramphenicol, ciprofloxacin and olaquindox in E. coli [14,15]. This OqxAB located plasmid has a high frequency of transfer among enterobacterial pathogens (Salmonella Typhimurium, Klebsiella pneumoniae, K. oxytoca sp. and Enterobacter aerogenes, etc) [14,16]. It is rarely known how E. coli develop or acquire the QdNO’s resistance. For the concern of animal and human safety, it is required to be elucidated whether the selection pressure of QdNOs contributes the emergence of OqxAB and whether the resistant determinants would spread to other enterobacterial pathogens. During the development of QdNOs resistance, it is an urgent need to explore the novel molecular mechanisms involved in the emergency and transfer of QdNO’s resistance in pathogenic bacteria especially E. coli.

Since serial passage is a traditional way to predict the resistance development to some antibiotics [17], some QdNOs resistant E. coli strains selected by two-fold increasing concentration of olaquindox and cyadox in vitro and some clinical QdNOs-resistant E. coli strains isolated from swine farms in China where QdNOs have been used for a long time, were subjected to analyze the development of QdNOs resistance. Since conjugation is the most
important way of horizontal transfer of some antibiotic resistance
determinants in *E. coli* [10], the horizontal transfer of QdNOs-
resistant determinant was then assessed by conjugative experi-
ment. Since a prokaryotic SSH PCR cDNA subtraction approach
developed by De Long is a technique that can be used to identify
differentially expressed genes under given conditions [19], this
approach was consequently carried out to investigate the novel
mechanism beyond the OqpAB-mediated QdNOs resistance.

### Materials and Methods

**Drugs and Stock Solutions**

Olaquindox (OLA), mequindox (MEQ), ampicillin (AMP),
cefotiofur (CTF), tetracycline (TET), chlorotetracycline (CTE),
chloramphenicol (CHL), florfenicol (FFC), trimethoprim (TMP),
sulfamethoxazole (SMZ), ciprofloxacine hydrochloride (CIP) and
colistin sulfate (CS) were supplied by China Institute of Veterinary
Drug Control. Gentamicin (GEN), oxytetracycline (OTC), furazo-
dione (FZD) and rifampicin (RIF) were provided by National
Institute for the Control of Pharmaceutical and Biological
Products (Beijing, China). Carbazid (CAR) was purchased from
Sigma Chemical Co. (St. Louis, MO) and cyadox (CVA) was kept
in our lab. Stock solutions of the antimicrobials were prepared
following the guidelines of the Clinical and Laboratory Standards
Institute (CLSI, 2009) or the suppliers’ recommendations. Besides,
stock solutions of CVA, CAR and FZD were prepared in DMSO;
Stock solutions of OLA, MEQ and FFC were prepared in hot
water; Stock solutions of CTF were prepared in phosphate buffer
(pH 6.0, 0.1 mol/L). After vortexing, the solutions were sterilized
by filtration through 0.22 μm pore size filters (Millipore) before
storage in aliquots at −20°C. Solutions of ampicillin, tetracycline
and oxytetracycline were prepared fresh on each occasion.

**Strains**

The porcine *E. coli* 79–161 strain was purchased from China
Institute of Veterinary Drug Control (Beijing, China). *E. coli*
NK5449 (CGMCC NO. 1.1437) being resistant to rifampicin at
100 μg/ml and as conjugation recipient was purchased from
China General Microbiological Culture Collection Center
(CGMCC, Beijing). *E. coli* ATCC25922 used as quality control (QC)
strains was quoted from performance standard of furantoin.
The resistance breakpoint of OLA was defined as an
4-fold increase of the MIC value made to minimize suffering of animals.

**MIC Determination**

MIC was determined by agar dilution antimicrobial suscepti-
bility test according to the M7-A7 guideline of the Clinical and
Laboratory Standards Institute (CLSI, 2009). Briefly, the density of
*E. coli* suspensions was adjusted to equal the density of a 0.5
McFarland standard [1 × 10⁸ CFU/ml]. After 10 fold dilution by
MH broth, 1–2 μL of the diluted suspension was inoculated on
prepared MH agar plates containing two-fold diluted concentra-
tion of each drug. The final concentration of strains was 10² CFU/
point on drug-containing plates. The standard strain of
*E. coli* ATCC25922 was used as quality control (QC) strain. Most of
the QC range and susceptible breakpoint were cited from CLSI
document M100-S19. The QC range of CIF was quoted from
performance standard of cefotaxime and ceftriaxone. The QC
range of FFC was quoted from performance standard of chloramphenicol. The resistance breakpoint of FZD (128 μg/ml)
was quoted from performance standard of furantoin. The
resistance breakpoints of CTF, FFC and CAR were cited from
published paper [12]. Olaquindox resistance has been defined
with a breakpoint of 64 μg/ml [1]. Acquisition of cyadox
resistance was defined as an ≥4-fold increase of the MIC value
comparing with its control parent strain [20].

**PCR Amplification of oqxA Genes**

Colonny PCR was proceeded to screen for oqxA genes in the selected *E. coli* strains. Based on the sequences of oqxA (GeneID
5962055) in *E. coli*, a pair of primers (OqxA-F 5’-GGGA-
TAGTTTTAACGGTGCAGTTG-3’ and OqxA-R 5’-TT
CAGGAGACGAGTTGTTG-3’) was designed to amplify
266-bp oqxA gene fragment. The amplification conditions com-
prised a denaturation step of 95°C for 5 min, followed by 32 cycles
of 94°C for 30 s, 64°C for 30 s, 72°C for 1 min and a final
extension for 10 min at 72°C. Amplions were sequenced by
Nanjing Genescript Biotechnology Limited Company (Nanjing,
China).

**Conjugative Transfer of QdNOs-resistant Determinant**

To investigate whether QdNOs-resistant determinant possess
horizontal transfer risk, filter conjugation was carried out
according to the method used by Yuan et al [21], in which
induced high-level QdNOs-resistant strains were used as donor
and *E. coli* NK5449 was used as recipient. The transconjugants
were selected on LB agar plates containing 100 μg/ml olaquindox
and rifampicin. The frequency of conjugation was calculated as

In vivo Isolation of Resistant *E. coli* from Swine

On swine farm in Wuhan Huaguoashan Livestock Company, six
groups of swine were breeding with drugs (Group 1 with no drug,
group 2 with 50 μg/ml olaquindox, group 3 with 50 μg/ml
chlorotetracycline, group 4 with 50 μg/ml cyadox, group 5 with
150 μg/ml cyadox and group 6 with 250 μg/ml cyadox). The
samples of anal swab were collected from each group of swine after
0, 45th and 100th day adding drugs to their basal diet. The *E. coli*
strains were isolated and identified by the selective agar
(MacConkey Agar) and classic biochemical test following the
microbiology laboratory guidebooks published by ministry of
health in China (2003). Species identification was confirmed using
an ABI 3130 system (Applied Biosystem, USA). 1~2 respective *E.
coli* strains selected from each of the six groups were subjected to
MIC determination as below. The experimental procedures
involving animals in the study were approved by the Animal
Care Center, Hubei Academy of Medical Sciences. All efforts were
made to minimize suffering of animals.
following formula: CFU of transconjugant/CFU of donor. The donor, recipient and transconjugant were identified by comparison of their EcoRI RiboPrint® pattern with that in identification database [22]. Comparing to E. coli NK5449, the similarity of the RiboPrint® pattern was shown in percent.

Preparation of mRNA

Before isolation of total RNA, driver and tester stains were grown in 50-ml drug-free Luria-Bertani (LB) broth till log phase. Briefly, overnight cultures were 100 fold diluted in 50-ml of LB broth, the culture were grown at 37°C with 250 rpm shaking. The density of culture reached to 0.6–0.8 OD₆₀₀, the cells were harvested by centrifugation of 1-ml aliquots in a microcentrufuge for 2 min. An RNeasy Protect Bacteria Mini Kit (Qiagen, Valencia, CA, USA) was used to lyse the cells and extract total RNA. The RNA was quantified by a light absorption at 260/280 nm. The purity and integrity of the RNA samples were verified by an electrophoresis in agarose gels using the 16S and 23S ribosomal bands as indicators. Samples of valid RNA preparations would fail to yield a product.

The mRNA was isolated using a MICROBExpress bacterial mRNA enrichment kit (Ambion, Austin, TX, USA). Every 5 preparations for each substrate were pooled before ethanol precipitation to obtain at least 2 μg mRNA per preparation. The mRNA quantity was measured by absorbance at 260 nm, and quality was assessed on agarose gel.

Prokaryotic SSH PCR cDNA Subtraction

Prokaryotic SSH PCR cDNA Subtraction

cDNA synthesis, cDNA digestion, adaptor ligation, first and second hybridization, suppression PCR and clone screening were carried out following the protocols of De Long with slight modification on the PCR-select cDNA subtraction kit(Clontech) [19].

A RNA polymerase sigma factor rpoE (the primer in Table 1) was used to detect the quality of double-strand cDNA. The amplification conditions comprised a denatured step of 95°C for 5 min, followed by 32 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 1 min and a final extension for 10 min at 72°C. Products were resolved by gel electrophoresis on a 2% (wt/vol) agarose gel. After second-strand cDNA synthesis, two cDNA synthesis reaction mixtures were pooled and purified with DNA fragment purification kit (Takara, Kyoto, Japan). Tester and driver cDNA were digested with Rsa I and purified also with DNA fragment purification kit (Takara).

To determine the efficiency of adapter ligation and subtraction, PCR amplification were performed according to the protocol in the PCR-Select cDNA subtraction kit by use of a housekeeping gene ribosomal protein S1 (rpsA) (the primer in Table 1). The amplification conditions comprised a denatured step of 95°C for 5 min, followed by cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final extension for 10 min at 72°C. For test

| Gene | Forward and reverse primer sequences | Melting temperature (°C) | Reference |
|------|-------------------------------------|--------------------------|-----------|
| 16S rRNA (rrsC) | 5’-GCTACAATGGCCGCATACAA-3’ | 55.9 | [69] |
| rpoE | 5’-CTGGTTTCCCCGTATGT-3’ | 51 | This study |
| rpsA | 5’-CTGACGCAAGTAGAAGCGG-3’ | 60 | This study |
| mobA | 5’-TGTATCCCGCGCAAGAGAAASA-3’ | 56.7 | This study |
| rpoA | 5’-GCAAGTTAGCGAGGAGCAAGC-3’ | 62.0 | This study |
| rplF | 5’-AACACCCGATAACATTGAGT-3’ | 56.9 | This study |
| fuaA | 5’-GTCGACGAGACGACCTT-3’ | 59.1 | This study |
| fabl | 5’-GCCACCGAGAGGTCGCTT-3’ | 56.4 | This study |
| cpX | 5’-AACCGCCCTGACCAAGC-3’ | 54.8 | This study |
| atpD | 5’-CACAGGTTGGAAGCAGATGT-3’ | 59.1 | This study |
| wbB | 5’-GGCCAGTTTACACGACA-3’ | 56.9 | This study |
| lolC | 5’-CTCCGGTTTCTGATGCTTC-3’ | 63.5 | This study |
| secA | 5’-CTCCGGTTTCTGATGCTTC-3’ | 54.0 | This study |
| flc | 5’-GACGCTTTTACTGTTTCG-3’ | 59.1 | This study |

Table 1. Primers used in SSH PCR cDNA subtraction and RT-qPCR.
of ligation efficiency, the cycles were 32. And for test of subtraction efficiency, the cycles were 18, 23, 28, 33 and 38.

After the PCR adaptor screen, 1, 2-clones were selected and submitted to Nanjing Genesis Biotechnology Limited Company (China) for sequencing directly. The sequences were aligned to available sequences in NCBI library by blastn algorithm (www.ncbi.nlm.nih.gov; BLAST). The homologous genes were subjected into molecular annotation system (http://www.capitalbio.com/support/mas) or KEGG library for gene function annotation and pathway analysis.

Reverse Transcription, Quantitative Real-time PCR (RT-qPCR)

4~5 μg of DNase-treated total RNA (isolated as described above) was used to synthesize cDNA according the method of De Long [19]. Real-time PCR was carried out on Bio-Rad iQ<sup>TM</sup> Multicolor Real-Time PCR Detection System, using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Takara) in a total volume of 25 μl: 12.5 μl SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup>, 1.0 μl template cDNA, 0.5 μl (final concentration 0.2 μM) or 0.25 μl (final concentration 0.1 μM) of each primer, and PCR-grade water to a final volume of 25 μl. Samples without template were set as negative control. At least 3 replicates were analyzed for each gene. Primer sequences were

Samples without template were set as negative control. At least 3 replicates were analyzed for each gene. Primer sequences were presented in Table 1. The fold change of selected genes was normalized to fold change of reference gene (16S rRNA gene). The relative expression was calculated according to the formula $2^{-\Delta\Delta Ct}$ [23]. The data were considered for significant differences by one-way ANOVA using SPSS programme ($P$$\leq$0.05).

Results

Resistance Phenotype of Selected Strains

Six representative strains (79O4-1, 79O4-2, 79O4-3, 79C4-1, 79C4-2 and 79C4-3) were obtained after stepwise selection by two-fold ascended concentration of olaquindox and cyadox. The variations of MICs during the selection procedure were presented in Table 2. Before selection, strains were susceptible to all the antibiotics tested. After 20 passages, the MICs of growth-control strains (79~20) have no significant change. High-level olaquindox resistant E. coli strains (79O4-1, 79O4-2 and 79O4-3) with MIC $\geq$256 μg/ml were selected under the pressure of olaquindox. Cyadox resistant E. coli strains (79C4-1, 79C4-2 and 79C4-3) with MIC $\geq$128 μg/ml were obtained by stepwise selection of cyadox. Notably, E. coli strains selected by olaquindox exhibited reduced susceptibility to ampicillin, tetracycline, oxytetracycline, chloramphenicol, florfenicol, trimethoprim and ciprofloxacin (see Table 2). All of the three strains (79O4-1, 79O4-2 and 79O4-3) selected by olaquindox were cross-resistant to the four QdNOs tested, while the three strains (79C4-1, 79C4-2 and 79C4-3) selected by cyadox were only resistant to carbadox and cyadox. Compared to cyadox, olaquindox might provide more potential in selection of multidrug resistance and cross-resistance in E.coli.

Before and after adding olaquindox, chlorotetracycline and cyadox into swine diet, 21 representative E.coli strains were isolated from 6 groups of swine by anal swab. From the data shown in Table 3, there is no significant change of the MIC before and after usage of QdNOs. Before usage of drug, the 7 E.coli strains selected at 0 day exhibited resistance to olaquindox with MIC $\geq$64 μg/ml. Notably, the 3 E.coli strains from the control group is naturally resistant to olaquindox with MIC = 64 μg/ml or 256 μg/ml.

**opxA** Gene Mediated Resistance

All of the in vitro selected strains presented in Table 2 were negative for opxA gene products, indicating that other mechanisms except for OpxAB mediated the resistance in the selected strains. However, 95% (20/21) E.coli strains isolated from swine farms were positive for opxA gene (see Table 3). The opxA positive strains are isolated before and after the usage of QdNOs drugs on pig farm. No relationship can be observed between the occurrence of opxA gene and usage of QdNOs drugs. The **opxA** gene positive strains always exhibited resistance to olaquindox except for E100-4 strain which carried **opxA** gene but is susceptible to olaquindox (MIC = 32 μg/ml).

Frequency of Conjugal Transfer

Frequencies of conjugal transfer for each strain were exemplified in Table 4. The conjugation frequencies of the three E. coli donors were at $10^{-10}$ CFU/donor. Before conjugation, the recipient is susceptible to the four QdNOs but resistant to rifampicin, the donors are resistant to the four QdNOs but susceptible to rifampicin. After conjugation, the transconjugants were resistant to both four QdNOs and rifampicin (see Table 4). The recipient and transconjugants share similar Riboprint<sup>®</sup> pattern with the similarity $>90$% (see Table 4), confirming that QdNOs-resistant determinant had been horizontally transferred by conjugation in E. coli strains.

Differentially Expressed Genes Determined by Prokaryotic SSH PCR cDNA Subtraction

By prokaryotic SSH PCR cDNA subtraction, 44 unique fragments were found in the QdNOs resistant strain E. coli 79O4-2 comparing to its control strain E. coli 79~20. Among these 44 genes, 3 sequences were with no significant similarity found in the Genbank; 40 sequences were found in genome of E. coli; 1 sequence was found in plasmid of E. coli (mobK) (see Table 5). The homology search results showed that QdNOs-resistant E. coli alters the expression of 18 genes involved in biosynthesis, 11 genes associated with metabolism, 3 genes encoding transporters, 3 genes involved in flagellar assembly, 1 gene encoding mobilization protein and 5 genes associated with hypothetical proteins (see Table 5).

In the 18 genes involved in biosynthesis, the number 1 gene encodes a iron-sulfur cluster binding protein, the number 2 gene (narH) encodes a nitrate reductase I. The number 3 to 12 genes (tpaA, trmA, trnA, glyS, ileS, rplFCX, rpsH and fucA) encode RNA polymerase, tRNA-methyltransferase, tRNA synthase, tRNA synthase and protein chain elongation factor respectively. The number 13 to 18 genes (lipA, lipX, tufM, fabI, otsK and pyrG) are related to biosynthesis of lipidate, lipid A, enterobacterial common antigen, fatty acid, trehalose and cytidine triphosphate respectively (see Table 5 and Figure 1).

In the 11 genes involved in metabolism, the number 19 and 20 genes (gpmA and pgp) participate in glucose degradation, the number 21 to 24 genes (cpxA, cpxA, pepN and fkpB) take part in degradation of damaged protein or protein modulation, the number 25 to 29 genes (napA, napD, napG, wbpB and yeqC) contribute to electron transport and ATP biosynthesis (see Table 5 and Figure 1).

As shown in table 5, the 3 transport proteins include spermidine/putrescine ABC transporter membrane protein (potC), outer membrane-specific lipoprotein transporter subunit (boIC) and preprotein translocase (secA). The flgD, flhA and flhC are involved in assembly of flagellar (see Table 5). The mobA gene is associated with the mobilization of bacterial (see Table 5).
Table 2. The MICs to 16 antibiotics of the strains selected by two-fold ascended concentrations of olaquinodox or cyadox in vitro.

| Strains | MIC to different antibiotics (µg/ml) | AMP | CTF | GEN | TET | OT | CHL | FFC | TMP | SMZ | FZD | CIP | CS | OLA | MEQ | CAR | CYA |
|---------|-------------------------------------|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 79O4-1  | selected by olaquinodox             | 16* | 1   | 0.25| 64  | 32 | 8   | 8   | 8*  | 8   | 0.031| 1   | 256 | 128 | 64  | >128|    |
| 79O4-2  |                                    | 16* | 2*  | 0.25| 64  | 32 | 32  | 8*  | 8   | 0.031| 1   | >256| >128| 128 | >128|    |
| 79O4-3  |                                    | 8   | ≤0.5| 0.5*| 64  | 32 | 8   | 8   | 4*  | 16  | 0.031| 1   | 256 | 128 | 32  | >128|    |
| 79C4-1  | selected by cyadox                  | 2   | ≤0.5| 0.25| 32  | 32 | 2   | 2   | 1   | 8   | 0.015| 1   | 32  | 32  | 64  | >128|    |
| 79C4-2  |                                    | 2   | ≤0.5| 0.25| 32  | 32 | 4   | 4   | 1   | 8   | 0.015| 1   | 16  | 16  | 8   | >128|    |
| 79C4-3  |                                    | 4   | ≤0.5| 0.5*| 32  | 32 | 8   | 8   | 1   | 8   | 0.015| 1   | 32  | 32  | 64  | >128|    |
| Growth control strain |                            | 79-20 | 0.125 | 2   | 2   | 4   | 4   | 1   | 8   | 0.008| 1   | 16  | 16  | 16  | 32  |     |    |
| QC standards  |                            | ATCC25922 | 0.125 | 0.5 | 2   | 2   | 2   | 4   | 4   | 8   | 0.008| 1   | 8   | 4   | 4   | 16  |     |    |
| QC range |                            | 2–8  | 0.03–0.12 | 0.25–1 | 0.5–2 | 0.5–2 | 2–8 | 2–8 | 0.5–2 | 8–32 | 4–16 | 0.004–0.015 | 0.25–1 | NA  | NA  | NA  |     |    |
| Breakpoint |                            | 32   | 8   | 16  | 16  | 16  | 32  | 16  | 16  | 512  | 128  | 4   | NA  | 64  | 4MIC | 32  | 4MIC |    |

Note: * MIC ascends 4-fold or more, but not reaching breakpoint; # MIC reach breakpoint. NA Not available.
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Table 3. Olaquinox MICs and oqxA containing in the clinical strains isolated from pigs before and after usage of growth promoters.

| Time for drug addition (days) | Group | Usage of Drugs and concentration in swine diet | MIC (µg/ml) | Olaquinox MIC (µg/ml) | OqxA (+positive, - negative) |
|------------------------------|-------|-----------------------------------------------|-------------|-----------------------|-------------------------------|
| 0 day                        | 1     | control                                       | E0-1        | 64                    | +                             |
|                              | 2     | 50 µg/ml olaquinox                            | E0-2        | 64                    | +                             |
|                              | 3     | 50 µg/ml chlorotetracycline                    | E0-3        | 128                   | +                             |
|                              | 4     | 50 µg/ml cyadox                               | E0-4        | 64                    | +                             |
|                              | 5     | 150 µg/ml cyadox                              | E0-5        | 128                   | +                             |
|                              | 6     | 250 µg/ml cyadox                              | E0-6-1      | 128                   | +                             |
|                              | 6     | 250 µg/ml cyadox                              | E0-6-2      | 128                   | +                             |
| After 45 days                | 1     | control                                       | E45-1       | 256                   | +                             |
|                              | 2     | 50 µg/ml olaquinox                            | E45-2       | 256                   | +                             |
|                              | 3     | 50 µg/ml chlorotetracycline                    | E45-3       | 128                   | +                             |
|                              | 4     | 50 µg/ml cyadox                               | E45-4       | 256                   | +                             |
|                              | 5     | 150 µg/ml cyadox                              | E45-5       | 128                   | +                             |
|                              | 6     | 250 µg/ml cyadox                              | E45-6-1     | 64                    | +                             |
|                              | 6     | 250 µg/ml cyadox                              | E45-6-2     | 64                    | +                             |
| After 100 days               | 1     | control                                       | E100-1      | 256                   | +                             |
|                              | 2     | 50 µg/ml olaquinox                            | E100-2      | 256                   | +                             |
|                              | 3     | 50 µg/ml chlorotetracycline                    | E100-3      | 128                   | +                             |
|                              | 4     | 50 µg/ml cyadox                               | E100-4      | 32                    | +                             |
|                              | 5     | 150 µg/ml cyadox                              | E100-5      | 64                    | -                             |
|                              | 6     | 250 µg/ml cyadox                              | E100-6-1    | 128                   | +                             |
|                              | 6     | 250 µg/ml cyadox                              | E100-6-2    | 256                   | +                             |

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Table 4. Transfer frequency and specific assessment of conjugation.

| Background strains | MICs to QdNOs (ug/ml) a | Similarity of Riboprint patterns between donor and transconjugant (%) b |
|--------------------|-------------------------|---------------------------------------------------------------------|
|                    | OLA | MEQ | CAR | RIF | OLA | MEQ | CAR | RIF |
| Recipient NK5449   | 256 | 128 | 8   | 8   | 200 | 100 |     |     |
| Donors 79O4-1      | 256 | 128 | 64  | 64  | >200| >200|     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |
| Donors 79O4-2      | 256 | 128 | 32  | 32  | >200| >200|     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |
| Donors 79O4-3      | 256 | 128 | 128 | 128 | >200| >200|     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |
| Transconjugant 79O4-1/N | 4.0 | 10  | 256 | 256 | 200 | 92  |     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |
| Transconjugant 79O4-2/N | 5.4 | 10  | 256 | 256 | 200 | 93  |     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |
| Transconjugant 79O4-3/N | 2.5 | 10  | 256 | 256 | 200 | 93  |     |     |
|                    | 128 | 128 | 128 | 128 | 128 | 128 |     |     |

Note: a-the transfer frequency was calculated by dividing the CFU of transconjugant by the CFU of donor; b-and c-the specificity of conjugation was assessed by the change of MIC and riboprint patterns of the recipient, donors and transconjugants.

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Discussion

Resistance Development Under QdNOs

Although the resistant E. coli strains were obtained from step-wise selection by QdNOs drugs in vitro, there is no significant change of susceptibility of the E.coli strains isolated from pigs farm before and after the usage of QdNOs drugs. It was revealed that the incidence and level of olaquindox resistance in the coliform floras isolated from pigs and pig farms was complex and largely fluctuated, the possible origin of olaquindox-resistant strains may be related to the biotypes of the drug-resistant sub-populations [24]. During the usage of olaquindox as feed additive on commercial pig farms from 1982 to 1984, the monitoring result about the development of olaquindox resistance showed that there was a low but not significant increasing incidence and level of resistance to olaquindox on drug-using farms [25]. Conclusively, it is unreliable to connect the emergency of QdNOs resistance to the usage of QdNOs drugs only by series passage in vitro. The resistance of E.coli strains isolated from the control group of pigs may due to the complexity of clinical environment.

Compared the MICs of QdNOs in the three strains (79O4-1, 79O4-2 and 79O4-3) selected by olaquindox to the three strains (79C4-1, 79C4-2 and 79C4-3) selected by cyadox, olaquindox appears to possess more potency in selection of cross-resistance to QdNOs, suggesting that long-term use of cyadox could be safer than olaquindox. The difference in the development of cross-resistance induced by olaquindox and cyadox may attribute to the discrepancy in the chemical characteristics or structure of these two drugs [2].

Similar to previous studies of resistant phenotype in E.coli strains isolated from pigs and pig farms, the QdNOs resistant strains selected in our study also exhibited multi-drug resistance against tetracycline and chloramphenicol [24,25,26]. Both the result of previous studies and our study revealed that multidrug resistance induced by olaquindox and cyadox may due to the complexity of clinical environment. However, different with previous studies, the six QdNOs resistant strains selected by in vitro passage ((79O4-1, 79O4-2, 79O4-3, 79C4-1, 79C4-2 and 79C4-3) were negative in multidrug efflux pump OqxAB [14,15]. The OqxAB pump (data not shown ) and showed much lower frequency of horizontal transfer (see table 4) with the ratio of 2.5~4.0×10^{-10} CFU/donor, suggesting that other mechanism beyond OqxAB might confer the multidrug resistance and lower transfer risk in the selected strains in our study. A prokaryotic SSH PCR cDNA subtraction was thus carried out to find the novel molecular mechanism involved in QdNOs resistance.

Differently Expressed Genes Involved in the QdNOs Resistance

Based on networks of the drug-target induced cell death pathway found recently [27], it is supposed that the QdNOs
Table 5. The differentially expressed fragments identified in the QdNOs resistant strain *E. coli* 7904-2.

| No. of sequence | Length(bp) | Encoded protein | Gene |
|-----------------|------------|-----------------|------|
| **Involved in biosynthesis** | | | |
| 1 | 435 | Iron-sulfur cluster binding protein | - |
| 2 | 176 | Nitrate reductase 1, beta (Fe-S) subunit | *narH* |
| 3 | 192 | RNA polymerase, alpha subunit | *rpoA* |
| 4 | 88 | tRNA (guanine-1-)-methyltransferase | *trmD* |
| 5 | 112 | tRNA pseudouridine synthase A | *truA* |
| 6 | 147 | Glycine tRNA synthetase, beta subunit | *glyS* |
| 7 | 123 | Isoleucyl-tRNA synthetase | *ileS* |
| 8 | 150 | 50S ribosomal subunit protein L6 | *rplF* |
| 9 | 126 | 50S ribosomal subunit protein L3 | *rplC* |
| 10 | 343 | 50S ribosomal subunit protein L24 | *rplK* |
| 11 | 264 | 30S ribosomal subunit protein S8 | *rpsH* |
| 12 | 406 | Protein chain elongation factor | *fusA* |
| 13 | 200 | Lipoate synthase | *lpA* |
| 14 | 233 | UDP-3-O-acyl N-acetylglucosamine deacetylase | *lpxC* |
| 15 | 54 | UDP-N-acetyl-D-mannosaminuronic acid transferase | *rfm* |
| 16 | 170 | Enoyl-(acyl-carrier-protein) reductase, ADH-dependent | *fabL* |
| 17 | 413 | Trehalose-6-phosphate synthase | *otsA* |
| 18 | 66 | CTP synthetase | *pyrG* |
| **Involved in metabolism** | | | |
| 19 | 252 | Phosphoglyceromutase 1 | *gpmA* |
| 20 | 473 | Glucosephosphate isomerase | *pgi* |
| 21 | 397 | ATP-dependent Clp protease, ATP-binding subunit | *clpX* |
| 22 | 161 | ATP-dependent Clp protease ATP-binding subunit | *clpA* |
| 23 | 90 | Aminopeptidase N | *pepN* |
| 24 | 137 | Peptidyl-prolyl cis-trans isomerase | *fkpB* |
| 25 | 238 | F1 sector of membrane-bound ATP synthase, alpha subunit | *atpA* |
| 26 | 456 | F1 sector of membrane-bound ATP synthase, beta subunit | *atpD* |
| 27 | 364 | F1 sector of membrane-bound ATP synthase, gamma subunit | *atpG* |
| 28 | 271 | 2-octaprenylphenol hydroxylase | *ubiB* |
| 29 | 161 | Isochorismatase hydrolase | *ycD* |
| **Involved in transport** | | | |
| 30 | 170 | spermidine/putrescine ABC transporter membrane protein | *potC* |
| 31 | 441 | outer membrane-specific lipoprotein transporter subunit; membrane component of ABC superfamily | *ioIC* |
| 32 | 160 | Preprotein translocase, SecA subunit | *secA* |
| **Flagellar assembly** | | | |
| 33 | 232 | Flagellar hook assembly protein | *flgD* |
| 34 | 246 | Flagellar biosynthesis protein FlhA | *flhA* |
| 35 | 544 | Flagellin | *flIC* |
| **Mobilization protein** | | | |
| 36 | 262 | Mobilization protein MobA | *mobA* |
| **Predicted protein** | | | |
| 37 | 111 | Putative enzyme | - |
| 38 | 191 | Predicted peptidoglycan peptidase | *yisX* |
| 39 | 226 | Conserved hypothetical protein | *ystN* |
| 40 | 163 | Conserved hypothetical protein | *yrbL* |
| 41 | 325 | Conserved hypothetical protein | *ybjP* |

Note: – No designated gene name.
Figure 1. The hypothetical mechanism of cell death induced by QdNOs and genes involved in the development of Quinoxaline resistance. The primary drug (red triangle)-target (unknown target) interaction initiate a metabolic feedback dependent on tricarboxylic acid (TCA) cycle, stimulate the oxidation of NADH through the electron transport chain, promote superoxide (O2•−) formation, damage the structure of Fe-S clusters, lead to the formation of hydroxyl radicals (OH•); and finally damage DNA, lipids or proteins and contributes to the cell death. The overexpressed genes found in our study (in the box and cycle) were involved into this process. The overexpression of some genes were induced in this drug-target cell death pathway (The dotted arrow), the others may inhibit the formation free radicals or prevent the damage by free radical (dotted line with a bar).

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Figure 2. Fold change of 11 up-regulated genes determined by RT-qPCR. The X-axis was the 11 representative genes (mobA, rpoA, rplF, fusA, fabI, clpX, atpD, ubiB, lolC, secA and fliC) which were selected from all the 44 differentially expressed genes. The Y-axis was the relative fold change of selected genes determined by RT-qPCR. The data were considered for significant differences by one-way ANOVA using SPSS program (P<0.05).

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binding to unknown target may initiate a metabolic feedback dependent on the tricarboxylic acid (TCA) cycle, stimulate the oxidation of NADH through the electron transport chain; promote superoxide (O$_2^-$) formation; damage the structure of Fe-S clusters, lead to the formation of hydroxyl radicals (OH$^-$); and finally damage DNA, lipids or protein and contributes to the death of E. coli (see Figure 1).

In the differently expressed genes, the iron-sulfur cluster binding protein encoding gene (gene number 1) participates in Fe-S cluster assembly. The nitrate reductase 1 encoding gene (narH) contribute to the electron transfer for Fe-S cluster [28,29]. Thus, overexpression of number 1 and 2 genes (narH) may compensate Fe-S cluster damage by assembly of Fe-S cluster (see figure 1).

The $spaA$ is a DNA-direct RNA polymerase which can interact with other proteins (ppIF, $spfC$, $spfX$, $spaH$ and others) and plays importance role in transcriptional regulation [30,31]. The $traD$, $trcA$, $gybS$ and $tlbS$ are essential enzymes for RNA modification and aminoacyl-tRNA synthesis which also play essential role on the protein synthesis [32]. The $fasA$ gene encoding for a translational regulator which is essential for translational elongation [33,34]. The overexpression of the translational regulator $fasA$ and some ribosomal proteins involved in translational process may compensate protein synthesis for oxidative damage by QdNOs and contribute to the multidrug resistance against some protein target drug of tetracycline and chloramphenicol.

The number 13 gene (lfpA) encodes lipoate synthase (LS) which is involved in the biosynthesis of Lipoic acid [35,36,37]. Lipoic acid is a protein-bound dithio-containing cofactor which is commonly required for the energy metabolism in E. coli [37]. Recent studies also show that lipoic acid is a free radical scavenger and a potent antioxidant [38,39,40]. The number 17 gene (atsAB) encodes trehalose-6-phosphate synthase which is responsible for trehalose biosynthesis [41,42,43]. It has been shown that trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold or oxidation [42,43,44]. The accumulation of trehalose can protect S. cerevisiae from oxidative damage and enhance resistance of bacterial to H$_2$O$_2$, accumulation of trehalose can protect desiccation, dehydration, heat, cold or oxidation [42,43,44]. The number 19 gene (phosphoglyceromutase 1, pgm1) encodes trehalase which may protect QdNOs-resistant E. coli [45]. Therefore, the overexpression of lfpA and atsA gene may promote production of two free radical scavenger (lipoic acid and trehalose) which may protect QdNOs-resistant E. coli from oxidative damage and result in QdNOs-resistance. There is no significant change of the expression of osyR or ssoRS regulon, so this mechanism of antioxidative protection mediated by lipoic acid and trehalose may be independent on the regulation of osyR or ssoRS [46].

The number 19 gene (phosphoglyceromutase 1, pgm1) and number 20 gene (glucose-6-phosphate isomerase, pgp) are involved in glycolysis which generates a small quantity of ATP and degrades glucose into pyruvocic acid and acetyl-CoA for tricarboxylic acid cycle (TCA) under aerobic growth condition. These two enzymes encoded by pgm1 and pgp gene are also important for central carbon metabolism (CCM) pathway which may influence the fidelity of DNA replication in E.coli [47,48]. The 2-octaprenylphenol hydroxylase (ubiB), F1 sector of membrane-bound ATP synthase $\alpha$ (atpA), $\beta$ (atpD) and $\gamma$ (atpG) subunit are involved in ATP biosynthesis, electron transport chain and oxidative phosphorylation [49].

Based on the function annotation and pathway analysis, the Clp, one of the key ATP-dependent proteases in E. coli, is required during some stress conditions [50,51,52,53]. The PepN, a major aminopeptidase in E. coli, can degrade amino acids into small molecular for TCA cycle [54]. The Clp protease cleave damaged proteins into large peptides and amino acids which are further degraded by PepN [55]. The fkbB, peptidyl-prolyl cis-trans isomerase, participates in several biochemical processes including protein folding, receptors signaling, protein trafficking and transcription [56]. Therefore, up-regulation of Clp proteases (21-ClpX and 22-ClpA), aminopeptidase pepN (number 23 gene) and protein folding chaperones fkbB (number 24 gene) may play important roles in regulating levels of specific proteins and in eliminating or modifying damaged or abnormal proteins [57].

Considering the drug-target induced cell death pathway and function analysis of the differentially expressed genes, the upregulation of some genes may be response for some process of cell death pathway, but others may compensate or inhibit that pathway. See figure 1, the two genes involved in glycolysis for TCA cycle (19-pgm1 and 20-pgp), four genes related to ATP biosynthesis and electron transport chain (25-atpA, 26-atpD, 27-atpG and 28-ubiB) and four genes associated with protein degradation and modulation (21-clpX, 22-clpA, 23-pepN and 24-fkbB) might be response for the metabolic feedback, hyperactivation of electron transport chain and protein damage, respectively. However, the two genes involved in Fe-S cluster assembly (atsAB gene and 2-narH) and several genes associated with protein biosynthesis may compensate the Fe-S cluster damage and protein damage respectively (see Figure 1). The overexpression of genes involved in biosynthesis of lipoic acid and trehalose (15-lipA and sotA) can eliminate superoxide formation and hydroxyl radical production (see Figure 1). The role of $fkbA$ and $ostA$ on clearance of free radical and development of QdNOs resistance would be investigated in our next plan, because unpublished data obtained in our lab recently revealed that QdNOs are endowed with producing free radical in E.coli cell.

Additionally, the potC gene is involved in spermidine uptake which plays an important role in bacterial growth including cell proliferation and differentiation [50]. The locC gene encodes an outer membrane-specific lipoprotein transporter which mediates the detachment of lipid-modified proteins and catalyzes release of outer membrane-directed lipoproteins from the inner membrane of E. coli [59,60,61]. Disruption of locCDE can prevent release of lipoprotein from inner membrane and shows to be lethal for E.coli [60]. The secA gene encodes a peripheral membrane ATPase which participates in preproteins translocation across the bacterial cytoplasmic membrane and plays an essential role in the survival of the bacterial species [62]. Recent study demonstrated that export of the superoxide dismutate SodA to periplasm was dependent on activity of SecA ATPase [63]. Therefore, overexpression of genes involved in transporters plays an essential role on the survival and growth of QdNOs-resistant E. coli. The lipA gene encodes zinc-dependent UDP-3-O-(acyl)-N-acetylgalactosamine deacetylase which catalyzes the first irreversible step of lipid A biosynthesis [64]. Lipid A biosynthesis (lipG), cytidine triphosphate biosynthesis (lipG), fatty acid biosynthesis (fadD) and enterobacterial common antigen biosynthesis (tfBM) may be essential for viability of E.coli. The $fkbD$, $fkbA$ and $fkbC$ genes are involved in flagellar assembly which exhibit functions in cell motility and sensibility [65]. Recent findings revealed that the flagellum participated in a variety of processes including biofilm formation, pathogenesis and even regulation of gene expression [58]. The soxRS gene encodes a mobilization protein which can be induced by some drugs and mediates the transfer of bacteriodes conjugation [66,67]. These genes may play important roles in physiological functions of QdNOs-resistant E. coli.
Conclusions

Results of the present study suggest that olaquindox possess more potency in selection of multi-drug resistant and cross-resistant E. coli strains. The induced QdNOs-resistant determinant shows lower frequencies of conjugation than previous reports in clinical isolates. More than 90% clinical isolates carry oprl gene, but it is negative in our in vitro selected QdNOs-resistant strains, indicating that there might be other mechanism conferring resistance. By SSH PCR subtraction, 44 genes were differentially expressed in the QdNOs-resistant strain 79O4-2 selected by olaquindox in vitro. The overexpression of genes involved in glycolysis for TCA cycle (gnd and pgf) and genes involved in ATP biosynthesis and electron transport chain (atpADG and ubiB) may due to the metabolic feedback and hyperactivation of electron transport chain, respectively. The genes involved in Fe-S cluster assembly (1-gene and narH) and genes involved in biosynthesis of lipic and trehalose (lipA and osA) may play an important role in clearance of free radical and prevention of Fe-S damage. The genes involved in protein biosynthesis (papD, trnD, track, glyS, iesF, spfE, spfC, spfX, spfH, spfF and fack) and genes involved in protein degradation and modulation may participate in compensation of the protein damage (see Figure 1). The other genes including genes encoding transporter, flagellar, lipid A and mobilization protein may be essential for the growth and physiological functions of QdNOs-resistant E. coli. Conclusively, most of these genes have previously been implicated in resistance or tolerance to superoxide stress, suggesting that their altered expression may be a part of a protective response in QdNOs-resistant E. coli [68]. Likewise, other identified changes in gene expression may affect the growth and transfer capacity of QdNOs resistance. Further studies are required to investigate the roles of these genes in development of QdNOs resistance by technique of gene knockout and transcription or proteomics. In addition, the molecular mechanism involved in the discrepancy of resistance development selected by olaquindox and cyadox is necessary to be investigated in future, because these information will be useful for the designation of new member of versatile QdNOs.

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Author Contributions

Conceived and designed the experiments: WG MD HH XW ZY. Performed the experiments: WG HW MY YS. Analyzed the data: WG HH MD DP ZY. Contributed reagents/materials/analysis tools: ZY YY LH ZL. Wrote the paper: WG HH MD ZY.

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