In Vitro characterization of colistin resistance and transfer of neomycin resistance in *Escherichia coli* O149 strains

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Abstract

Neomycin, an aminoglycoside, is use at farm level for *Escherichia coli* treatment of piglets postweaning diarrhea, with 40% of unsuccessfully treatment due to the antimicrobial resistance. To overcome this situation, veterinarians use colistin also call polymyxin E, but this antibiotic is not homologated in Canada, even if it seems to be effective against *E.coli*. The described resistance to polymyxin is associated to a modification of the LPS core and the lipid A regions in the bacteria. For *Salmonella*, those modifications are associated to the two components system PmrA-PmrB. This system is known in *E.coli* but not yet reported to be implicated in colistin resistance. The neomycin resistance is linked to enzymatic modifications associated to genes located on plasmids. This study has for objectives to investigate the acquisition of colistin resistance and study the transfer of neomycin resistance between *E.coli* strains and other enterobacteria. *E.coli* O149 strains isolated from clinical cases (2008 to 2011) were used and susceptibility testing of strains was performed by the disk diffusion method. E-test and a micro-dilution method were used to determine the minimal inhibitory concentration (MIC) of colistin and neomycin respectively. All tested strains had a MIC higher than 128 ppm for neomycin whereas MICs were between 0.064 to 0.128 ppm for colistin, indicating that all strains were resistance to neomycin but all susceptible to colistin. In these isolates, neomycin antimicrobial resistance genes *aac(3)-IV*, *apkA1* and *aphA2* have been detected using PCR. Conjugation experiments are presently being performed. Colistin natural mutants (n=22) were created through serial passages on LB agar with 25xMIC. Sequencing of the PmrA-PmrB region of these mutants was performed to identify mutations. Three PmrA and seven PmrB mutations were for the first time reported in *E.coli* O149. Others mutants are still under investigation for other possible resistance mechanism.

Introduction

*Escherichia coli* O149 is the major cause of post-weaning diarrhea with an important economic impact. Veterinarians use neomycin, an aminoglycoside for treatment of this condition. Unfortunately near 40% of the treatments are unsuccessfully due to *E.coli* resistance to this antibiotic (Maynard et al., 2003). The most frequently associated mechanism of resistance is by enzymatic modification, acetylation and phosphorylation, of the neomycin. Three genes are mostly associated with neomycin resistance; *acc(3)-IV*, *apk(3')-I*, *aph(3')-II* and most of the time, we find those genes on plasmids of different size (4 to 93kb) (Shaw et al., 1993). To overcome this situation, veterinarians use colistin sulphate (CS), a polypeptide antimicrobial also call polymyxin E, but this antibiotic is not authorize in Canada even if it seems to be effective against *E.coli*, because it is use in human medicine for Pseudomonas aeruginosa in kystic fibrosis. In other countries, this polypeptide antibiotic is recommended for the oral therapy of intestinal infections in pig. The target of CS is the Gram negative bacteria lipopolysaccharide (LPS) molecule by a cationic interaction leading to a displace of cation magnesium Mg²⁺ and calcium Ca²⁺ causing a leakage of intracellular contents and bacterial death (Wang and Quinn, 2010). Resistance to colistin have been reported in *Salmonella* sp. by the two components system PmrAB. This system is associated with a modification of the LPS by adding a L-ara4N group to LPS core causing a reduction of negative charge, leading to a less negative LPS charge (Boll et al., 1994). A mutation in those genes could lead to a constitutively of the system leading to a CS resistance by decreases the cationic liaison between CS and LPS. Therefore, the aim of this study was to first evaluate the *in vitro* acquisition of CS resistance and second to assess the *in vitro* transfer of neomycin resistance.
Material & method

Eight strains of E.coli O149 isolated from clinical cases (2008 to 2011) by ECL lab were selected for neomycin resistance. Susceptibility testing was performed by the disk diffusion method. Briefly, a dilution corresponding to a McFarland 0.5 was made in saline solution and uniformly plated on Mueller-Hinton (MH) agar, disks were aseptically added and plates were incubated overnight at 37°C. E-test was used to determine the MIC of colistin, briefly an uniformly plate was made with a dilution corresponding to a McFarland 0.5 on MH agar, E-test bands were aseptically added and plates were incubated overnight at 37°C. For neomycin MIC, a micro-dilution method was used. Briefly, in sterile 96-well polystyrene microtitre plates, serial double dilution were made in MH broth from 128 mg/L to 0.25 mg/L. Each well was inoculated with approximately 5x10^6 CFU/ml of E.coli strains in MH broth. The MIC was determined as the lowest concentration that resulted in inhibition of bacterial growth. In all MIC experiments, ATCC 25922 E.coli strain was used as control. For the 8 E. coli strains, PCR was used to determine the presence of antimicrobial resistance genes acc(3)-IV(F-GTGTGCTGCTGGTCCACAGC R-AGTTGACCCAGGGCTGTCGC), apk3'-I (F-ATGGGCTCGCGATAATGTC R-CTCACCGAGGCAGTTCCAT) and aph(3')-II (F-GAAACAGATGGATAGCCACGC R-GCTCTTTCAGCAATATCACGG).

Colistin mutants were created by incubation of each strain overnight at 37°C, on Luria-Bertani broth (LB) with a colistin concentration under the MIC. Serial passages were done on LB with 25xMIC for mutants selection. Mutant DNA was extracted by standard boiling method. Amplification of genes pmrA and pmrB was performed by PCR using respectively primers F-CAAACTTGCAGGAGAGTGAG R-GCTGATCAGCTCAAACCCCA and F-GGCTTGGCTATATGCTGGT R-TTAACTACCGTGTTCAGCGT. PCR products were purified with PureLink® PCR Purification Kit (Invitrogen, Canada). Sequencing was performed by Sanger (Genome Québec). Alignments of sequence were done with Clustal X software.

Results

For the first objective on antimicrobial resistance, all tested strains had a MIC higher than 128 ppm for neomycin whereas MICs were between 0.064 to 0.128 ppm for colistin, indicating that all strains were resistant to neomycin but all susceptible to colistin. PCR detection of neomycin resistance genes showed that apk(3')-I was found in six strains. Strains 1000 and 2000 have two resistance genes respectively acc(3)-IV, apkl(3')-I and acc(3)-IV, aph(3')-II. (Fig 1)

Mutants were created (n=24) and sequencing showed 7 different mutation sites in pmrAB region leading to a colistin resistance phenotype. Most of the mutations were found in mutants from E.coli ATCC 25922 strain and these mutations were observed in the pmrB gene. (Fig 2)
Discussion

Study of colistin resistance

This study is the first to report exact mutation in pmrAB genes leading to colistin resistance in *E.coli* O149 field strains and *E.coli* ATCC 25922 strain. In another study (Sun et al., 2009), pmrAB mutations were identified for *Salmonella* sp. In our study, mutants had higher MIC values (30 to 80x) comparatively to *Salmonella* mutants (2 to 35x) suggesting that the site of mutation in pmrAB is probably linked to the degree of resistance. We also create mutants without a pmrAB mutation suggesting another mechanism of resistance. Other genes have been reported to have a higher expression in a bile salt medium leading to colistin resistance (Kus et al., 2011). The arn operon genes which are involved in the synthesis and transport of the L-ara4N subunit (Wang and Quinn, 2010) and acrAB genes who are involved in a multidrug efflux system are potential other genes implicated in the colistin resistance without a pmrA or pmrB mutation.

Conclusion

This study showed mutations in pmrAB genes leading to colistin resistance in *E.coli* strains. A better understanding of the mechanism of resistance to colistin is needed for a better use of this antibiotic and avoid therapeutic practices having an impact of antimicrobial resistance of *E.coli* associated to postweaning diarrhea or other disease. Further studies are on going to characterized mutations not associated to pmrA / pmrB genes.

References


