Characterization of Efflux Pumps and its role on the Intrinsic Antimicrobial Resistance in Antimicrobial Resistant Salmonella enterica from Clinical and Food Samples

Ashraf A. Khan[1*]., Shaohua Zhao[2] and Mohamed S. Nawaz[1]

[1]Division of Microbiology, National Center for Toxicological Research, U. S. FDA, Jefferson, AR 72079. [2]Division of Animal and Food Microbiology, Office of Research, Center for Veterinary Medicine, U.S. Food and Drug Administration, Laurel, Maryland, USA

Ashraf.Khan@fda.hhs.gov
Office: 870-543-7601

Abstract

Dissemination of antibiotic resistance amongst pathogenic bacteria has been concern for public health. The impact of these drugs on selection of resistant bacteria and their subsequent drug resistance mechanisms are not well understood. In this study fourteen aminoglycoside-resistant Salmonella enterica isolated from clinical and food samples which carried the same resistance genes but had different MICs (minimum inhibitory concentrations) and were analyzed using whole genomic sequence analysis and assessing efflux pump activities with and without efflux pump inhibitors. Whole genome sequence analysis showed 10 strains had very high correlation with the presence of known resistant determinants. Ethidium bromide accumulation studies showed all strains have higher efflux activity as compared to susceptible Salmonella strains. To determine if carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a known efflux pump inhibitor has synergistic effect with either streptomycin or gentamicin on aminoglycoside resistant strains. Two kanamycin-resistant Salmonella strains with the largest change in MIC with CCCP are those that were found to have the strongest efflux pump systems. This may be indicative that for aminoglycosides having many active efflux pumps is a requirement to having high MIC. However, with the Folate pathway inhibitor resistant strains this trend appears to be reversed, strain 43788 which shows no change in MIC after CCCP addition appear to have the strongest active efflux pump system while strains N45955, N42472 and 43791 all appear to have efflux pump systems of equal strength. Further characterization of these isolates is in progress. Due to public health concern, it is important for regulators to limit the spread of MDR pathogens and to protect the efficacy of these life-saving antimicrobial drugs.

Introduction

Salmonella spp. are recognized as major foodborne pathogens among humans worldwide. Large amounts of antimicrobial agents are given to livestock for treatment and non-therapeutic purposes or to humans for treatment of infectious diseases. However, the impact of these drugs on induction and their contribution to the development of resistance are not well studied. Antimicrobial susceptibility testing of bacterial isolates is essential for clinical diagnosis, to detect emerging problems and to guide empirical treatment. Current phenotypic procedures in conjunction with whole-genomic sequencing (WGS) may soon be within reach even for routine surveillance of antimicrobial resistance compared with using only phenotypic methods. This study has shown the potential to use WGS to predict antimicrobial resistance in *Salmonella* isolates collected from human, animal and food isolates with various resistance patterns. The WGS data of *Salmonella* isolates showed high level of concurrence between MICs and presence of known resistance genes. However, despite the high level of concordance between genotypic and phenotypic methods, there was some disagreement specifically for aminoglycosides. Also, with presence of same resistance gene, there were wide range of MICs values difference in the different strains. We report in this efflux pump plays an important role in antimicrobial resistance which can't be predicted by WGS.

Results

Table 1. Genotype and phenotype comparison of Salmonella isolates from humans and retail meat

	No. of test results							
	Phenotype: resistant		Phenotype: susceptible					
Antibiotic	Genotype: resistant	Genotype: susceptible	Genotype: resistant	Genotype: susceptible	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Aminoglycosides								
GEN	99	6	5	530	94.3	99.1	95.2	98.9
STR	257	3	35	345	98.8	90.8	88.0	99.1
Beta-lactam/beta-lactam inhibitor								
AMC	114	2	0	524	98.3	100.0	100	99.6
Cephems								
FOX	93	2	21	524	97.9	96.1	81.6	99.6
TIO	113	0	4	523	100.0	99.2	96.6	100
CRO	116	0	1	523	100.0	99.8	99.1	100
Penicillin								
AMP	241	1	1	397	99.6	99.7	99.6	99.7
Folate pathway inhibitors								
FIS	244	1	0	395	99.6	100.0	100	99.7
SXT	19	3	0	618	86.4	100.0	100	99.5
Macrolide								
AZM	1	0	0	639	100.0	100.0	100	100
Phenicol								
CHL	44	0	1	595	100.0	99.8	97.8	100
Quinolones								
CIP	4	0	0	636	100.0	100.0	100	100
NAL	13	2	0	625	86.7	100.0	100	99.7
Tetracycline								
TET	349	0	0	291	100.0	100.0	100	100
Total	1,707	20	68	7,164	98.8	99.1	96.2	99.7

[a] Abbreviations: GEN, gentamicin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; AMP, ampicillin; FIS, sulfisoxazole; SXT, trimethoprim-sulfamethoxazole; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; PPV, positive predictive value; NPV, negative predictive value.

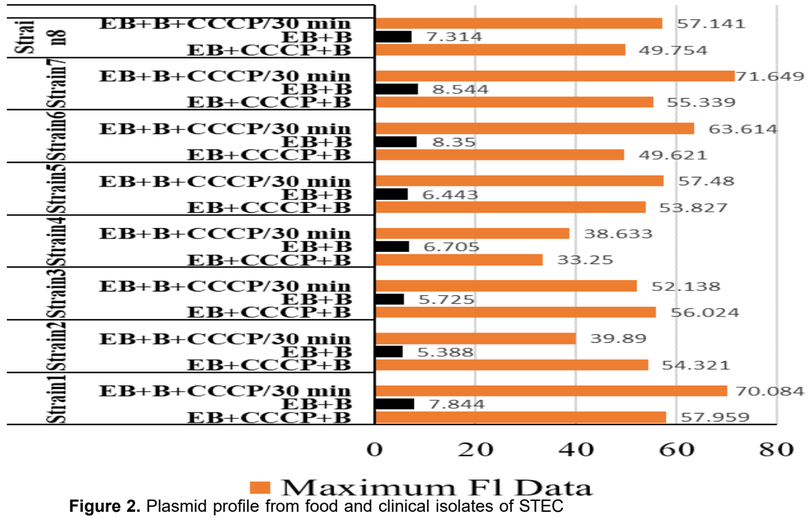


Figure 2. Plasmid profile from food and clinical isolates of STEC

Materials and Methods

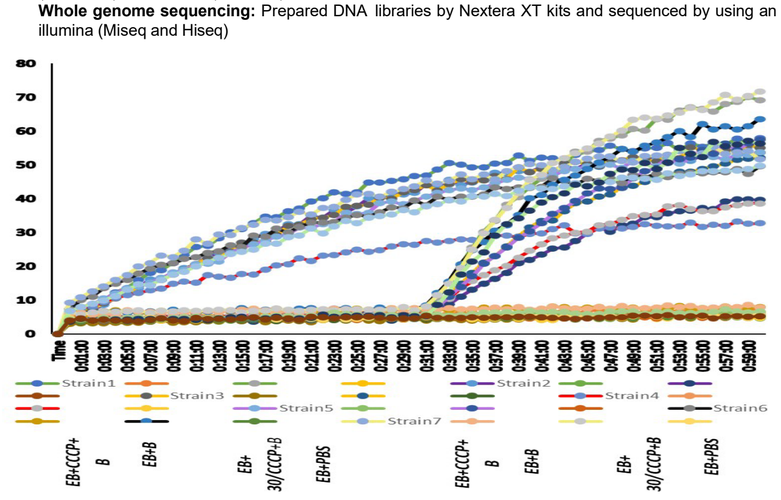
Bacterial strains : All food and clinical *Salmonella* isolates used in this study were obtained from CVM and FDA-Arkansas Regional Laboratory.

DNA isolation from bacteria: Total bacterial DNA was extracted using blood and tissue kit and according to the manufacture's protocol and stored at -20 °C until batch analysis.

Antimicrobial susceptibility: Antimicrobial susceptibility testing was performed for 14 antimicrobials, including gentamicin, streptomycin, ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, azithromycin, chloramphenicol, nalidixic acid, ciprofloxacin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline. MICs were determined by broth microdilution using dehydrated panels CMV2AGNF and CMV3AGNF (Thermo Fisher Scientific, Waltham, MA) according to standard protocols.

Efflux pump assay: The EtBr accumulation assay was performed to determine the efflux pump activity with and without inhibitor. Freshly grown bacterial were centrifuged, washed, and resuspended in 50 mM sodium phosphate buffer solution (pH 7.2). The cell suspensions were adjusted to an OD600 of 0.2. Suspensions (100 µl) of *Salmonella* cultures were placed in 96-well microtiter plates with EtBr (final concentration, 5 µg/ml). Carbonyl cyanide m-chlorophenylhydrazone (CCCP, 100 µM) was added to each well at 0.0 min and after 33 min. The relative fluorescence intensity was measured at 30s intervals up to 80 min using a fluorescence spectrophotometer (Spectra MAX Gemini EM, Molecular Devices, Sunnyvale, CA, USA) with wavelengths of 530 nm (excitation) and 600 nm (emission).

Whole genome sequencing: Prepared DNA libraries by Nextera XT kits and sequenced by using an illumina (Miseq and Hiseq)



Conclusion

- Most of the *Salmonella* isolates were resistant to one and more than one antibiotics, however, several strains were phenotypically resistant but genotypically sensitive.
- WGS of MDR *Salmonella* strains were analyzed for aminoglycosides, β-lactam, quinolones, tetracycline, macrolide and folate pathway inhibitors and results showed high concurrence between MICs and known resistance genes.
- MDR *Salmonella* strains from CVM and ORA were analyzed for antibiotic sensitivity and efflux activity using ethidium bromide with inhibitor and without inhibitor. Assessment of the efflux pump using efflux pump inhibitor and ethidium bromide showed a significant increase in the accumulation of ethidium bromide in *Salmonella* strains resistant to aminoglycosides.
- WGS is an important technique to understand the genetics of antibiotic resistances, however role of EP in those strains showing phenotypic resistance but genotypically negative .