ASSESSMENT OF EFFLUX ACTIVITY IN MDR *ENTEROCOCCUS* ISOLATES

Kanchan Jangir[1], Anjali Gangwal[2], S. Meharchandani and Krupa K. Soni[3]
Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner- 334 001, Rajasthan, India

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ABSTRACT

The following study was conducted with an objective of determining the efflux activity in multi-drug resistant (MDR) *Enterococcus* isolates obtained from healthy poultry faeces of Bikaner region. A total of 40 MDR *Enterococcus* isolates were investigated for the presence of efflux activity using ethidium-bromide cartwheel method which was further confirmed by using an efflux pump inhibitor CCCP (Carbonyl cyanide *m*-chlorophenyl hydrazone). Study demonstrated a high level of efflux activity as none of the isolates showed fluorescence at concentrations varying from 0.5-2.5 mg/ml Et-Br. But when added with CCCP, fluorescence was exhibited. Thus, efflux appeared as one of the possible causes of intrinsic multi-drug resistance in *Enterococcus* isolates. Hence it can be suggested that inhibition of the multi-drug efflux pumps would serve in potentially restoring the effectiveness of antimicrobial chemotherapy of infectious disease caused by bacteria that have multi-drug efflux pumps.

Key words: *Enterococcus*, MDR, efflux, efflux pump inhibitors

Introduction

The increasing multi-drug resistance reported in bacterial clinical isolates has become a major concern in the public health area given that the lack of treatment options for these infections, with the existing antibiotics, is becoming more and more problematic (Theuretzbacher, 2012). Antimicrobials, as essential feed additives, are an integral part of scientific animal farming both to treat clinical diseases and to increase productivity. Antibiotic usage remains one of the most important factors that promote the emergence, selection and dissemination of antibiotic resistant microorganisms in both veterinary and human medicine (Witte, 1998). It's very common practice in intensive poultry farming to administer antibiotics to whole flocks rather than individual animals through feed or drinking water at a sub therapeutic dose for use as a prophylaxis, metaphylaxis and growth promotion purposes which could potential lead to antibiotic resistance (Hart *et al.,* 2004). This selection pressure for resistance in bacteria in poultry consequently leads to harbouring of faecal flora that contain a relatively high proportion of resistant bacteria (Van Den Bogaard and Stobberingh, 1999).Thereby monitoring the prevalence of resistance in indicator bacteria like *Enterococcus* makes it practicable to evaluate the prevalence of resistance and to detect transfer of resistant genes from animals to humans and vice versa. Multidrug resistance is common among enterococci (Donabedian *et al.*, 2003). A major reason why these organisms survive in hospital environment is the intrinsic resistance to several commonly used antibiotics (cephalosporins, aminoglycosides, clindamycin, tetracyclines and erythromycin) and, perhaps more importantly, their ability to acquire antibiotic resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge for therapeutic measures (Coque *et al.*, 2008). MDR enterococci display a wide repertoire of antibiotic resistance mechanisms including modification of drug targets, inactivation of therapeutic agents, over expression of efflux pumps and a sophisticated cell envelope adaptive response that promotes survival in the human host and the nosocomial environment. One of the most likely mechanisms for intrinsic drug resistance in enterococci is active efflux (Lynch *et al.,* 1997). Multi-drug resistance can be the result of an over-expression of the bacterial efflux pump systems, used to extrude unrelated antibiotics prior to their reaching the intended targets (Piddock, 2006). A thorough understanding of the mechanisms underlying antibiotic resistance in enterococci is the first step for devising strategies to control the spread of these organisms and potentially establish novel therapeutic approaches.

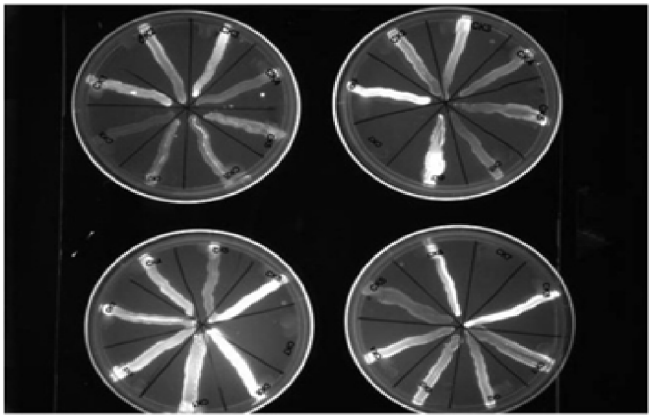

Fig. 1: Fluorescence of varying intensity produced by *Enterococcus* isolates at concentrations 1 to 2.5 mg/ml Et-Br along with CCCP on Nutrient agar plates at pH 8.

Materials and Methods

1) **Isolates:** A total of 40 *Enterococcus* isolates used in this

[#1]Part of M.V.Sc. Thesis of first author, present address: Incharge Veterinary Officer, Veterinary Hospital, Shri vijaynagar, Department of Animal Husbandry, Rajasthan, and Email: kanchanjangid87@gmail.com, Mob. No: 7665015201, [2]VO, Incharge Veterinary Hospital, Swaimadopur, Department of Animal Husbandry, Rajasthan, [3]Teaching Associate, Department of Veterinary Public Health, CVAS, Bikaner

study were pre-isolated and identified (Jangir *et al*., 2015).

2) **Identification of MDR isolates:** All the isolates were subjected to ABST and MDR isolates were identified on the basis of MAR Index (Jangir *et al*., 2015).
3) **Assessment of efflux activity**: It was conducted using ethidium bromide (EtBr), a common substrate of efflux pumps (Martins *et al*., 2011). The method is very simple, easy to perform and highly replicative. The methodology involved in the test is described below:

a) Nutrient agar plates were prepared (on the same day of experiment or on previous day) containing EtBr concentrations varying from 0.5 to 2.5 mg/l.
b) Overnight cultures of the enterococcal isolates to be tested are prepared in liquid media and in the following day their concentration was adjusted to 0.5 of a McFarland standard.
c) The NA plates were then divided into eight sectors by radial lines, forming a cartwheel pattern.
d) The adjusted bacterial cultures and a reference strain (*E. coli*) are then swabbed on the Et Br-NA plates starting from the center of the plate to the margin.
e) The NA plates are then incubated at 37ºC for 16 hours.
f) The minimum concentration of Et-Br that produces fluorescence of the bacterial mass was recorded.
g) Again the procedure is repeated for same isolates along with uncoupler CCCP (Carbonyl cyanide 3-choloro phenyl hydrazine) at the concentration of 6 µl for 1 ml broth and 120 µl for each NA-Et Br plates.
h) The plates were observed under UV illuminator for florescence and its inhibition after addition of CCCP.

Results and Discussion

Membrane bound multidrug resistance (MDR) efflux pumps are found in all bacteria and their primary functions could be other than antibiotic resistance, which include maintenance of intracellular solute concentrations or the cell homeostasis, extrusion of toxic byproducts of metabolism or transport of amino acids and nucleotides (Piddock *et al.*, 2006). Importantly, active multi-drug efflux is a major mechanism for bacterial pathogen drug resistance. Therefore, because of their overwhelming presence in bacterial pathogens, these active multi-drug efflux mechanisms remain a major area of intense study, so that ultimately measures may be discovered to inhibit these active multi-drug efflux pumps.

Efflux pump-mediated resistance to single or multiple antimicrobial agents has not only raised serious concerns but also has constricted the treatment options against bacterial infections (Nikaido, 1996). Efflux mechanisms of resistance, both drug specific and multidrug, are important determinants of intrinsic and/or acquired resistance to antimicrobials. Multidrug efflux mechanisms are broadly conserved in bacteria, are almost invariably chromosome encoded and their expression in many instances results from mutations in regulatory genes. In contrast, drug-specific efflux mechanisms are generally encoded by plasmids and/or other mobile genetic elements (transposons, integrons) that carry additional resistance genes, and so their ready acquisition is compounded by their association with multidrug resistance. Efflux pumps reduce the accumulation of antibiotics inside of the bacterial cells, and the slow phase in which the process of antibiotic efflux takes place provides sufficient time for the bacterium to adapt to the antibiotics and become resistant through mutations or alteration of antibiotic targets (Li and Nikaido, 2009). Further, the over-expression of efflux pumps enhances the resistances to antimicrobials (Okusu *et al.*, 1996). Based on the sequence comparison, efflux pumps are grouped into five major categories: The major facilitator superfamily (MFS), the adenosine triphosphate (ATP)-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion family (Paulsen *et al.,* 2003). Efflux pumps are now known to confer resistance to almost all classes of antibiotics (Morar and Wright, 2010). Efflux pumps for various drugs are found in enterococcal species as described in previous studies.

In the present study, as compared to *E. coli,* enterococcal isolates did not show fluorescence at given concentrations of Et-Br that indicated that there was no accumulation of Et-Br inside the cells which means a high degree of efflux mechanism was prevalent in isolates. This may be an explanation for multi-drug resistance in enterococcal isolates. Assessment of the efflux activity in all MDR enterococcal isolates was further evaluated after the addition of Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). CCCP causes an uncoupling of the proton gradient thus inhibit energy dependent efflux mechanism. Hence on addition of CCCP florescence must be observed due to accumulation of EtBr in isolates. But after several attempts, accumulation of EtBr was not appreciated in any of the enterococcal isolates. When pH was varied during the experiment and as it approached to 8, fluorescence was detected in isolates that indicated that EtBr has started accumulating at higher pH. This shows that CCCP in enterococcal isolates inhibit efflux mechanism at higher pH values. Similar observations were also detected in a study conducted by Spengler *et al.* (2009) on enterococcal isolates. His results showed that the intrinsic efflux system of *Enterococcus* strain is controlled by energy derived from the catabolism of glucose and the proton concentration of the medium. At pH 5, agents that inhibit efflux pumps in Gram-positive organisms and the proton gradient un-coupler CCCP do not increase accumulation nor inhibit efflux of Et-Br. In contrast, at pH 8, where the proton concentration is 1,000-fold lower, these agents increase accumulation and efflux of EB. These results are relevant to infections produced by *E. faecalis* and subsequent antibiotic therapy with antibiotics to which the organism is known to be intrinsically resistant.

Analyses of the structure-function relationships of single- and multi-drug efflux pumps may indentify key residues for discovery of efflux inhibitors. Therefore, identification of functionally conserved regions within efflux pumps should be useful for design of efflux pump inhibitors (Rahman *et al.,* 2008). Thus, inhibition of the multi-drug efflux pumps would aid in potentially restoring the effectiveness of antimicrobial chemotherapy of infectious disease caused by bacteria that have multi-drug efflux pumps. Therefore, morbidity and mortality frequencies may be diminished, because of multi-drug efflux inhibition (Lewis *et al*., 2001).

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