Sindh Univ. Res. Jour. (Sci. Ser.) Vol.49(3) 649-654 (2017)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Minimum Inhibitory Concentration Evaluator and Disc Diffusion Testing Techniques to Profile Antimicrobial Resistance in *Arcobacterbutzleri*

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Received 23rd November 2015 and Revised 26th October 2016

Abstract: The major objective of this study was to evaluate discrepancy in between two antibiotic susceptibility techniques, minimum inhibitory concentration evaluator (M.I.C.E) and disc diffusion. A total of 75*AButzleri*isolates were tested against ampicillin, tetracycline, ciprofloxacin, erythromycin, cefotaximeand gentamycin. The results revealed that 89.3% and 92% for ampicillin, 22.7% and 26.7% for ciprofloxacin,77.7% and 57.3% for erythromycin,9.4% and 13.3% for tetracycline, 53.3% and 73.3% for cefotaximeand 22.6% and 26.7% for gentamycin, isolates were resistant using M.I.C.E and disc diffusion, respectively. Moreover, multi-drug resistant was noticed in 16% by M.I.C.E and 12% by disc diffusion methods. Fisher's analysis showed that both methods tested were non-significant (P≥0.05) for other antibiotics except erythromycin (P=0.0144) and cefotaxime (p=0.0173). In conclusion, tetracycline can be used as a drug of choice to treat infections caused by *A. butzleri*and either of the methods, disc diffusion or M.I.C.E, can be used for four of the six antibiotics tested.

Keywords: *Arcobacterbutzleri*; Antimicrobial resistance; M.I.C.E and Disc diffusion.

1. INTRODUCTION

*Arcobacter*is recognized as a potential food and water borne pathogen. Nineteen species of *Arcobacter* have currently been isolated from a variety of animals, animal-derived food products, seafood, water and humans (Hsu and Lee 2015; Douidah *et al.*, 2014; Collado and Figueras, 2011; Shah *et al.*, 2011). Among these nineteen species, *A. butzleri* and *A.cryaerophilus* have been rated as serious hazards for human health (ICMSF, 2002).It has been isolated from animals, foods of animal origin, water and vegetables (Collado and Figueras, 2011) thus humans get infected if these foods are utilized uncooked or undercooked (Shah *et al.*, 2011a; Collado and Figueras, 2011).Human patients have shown the symptoms like gastroenteritis including abdominal pain, acute diarrhea or prolonged watery diarrhea for up to two months (Van den *et al.*, 2014; Vandenberg *et al.*, 2004). Underlying mechanisms of pathogenesis and immune response are unknown yet. The main clinical sign of disease caused by *Arcobacter* is diarrhea without blood which is considered as self-limiting (Vandenberg *et al.*, 2006). Antibiotic therapy depends on severity of disease and period of illness. The commonly prescribed drugs for treatment are erythromycin, ciprofloxacin, tetracycline, doxycycline and gentamycin (Collado and Figueras, 2011). Since the establishment of new genus *Arcobacter* till to date, various laboratory methodologies such as Epsilometer test (E-test), agar dilution, disc diffusion, and broth micro dilution (Collado and Figueras, 2011) have been applied to determine in-vitro susceptibility profiles of *Arcobacter* spp. Againsta range of antimicrobial agents but none of them have proved to be the gold standard. The lack of standard antimicrobial susceptibility methods for members of family *Campylobacteraceae* and the cumbersomeness of dilution methods have necessitated the work to be carried out to compare methods so as their antibiotic susceptibility profiles may be reported easily. The present study was conducted to report the prevalence of antimicrobial resistance in *A. butzleri* and to compare the efficiency of two agar diffusion based methods, disc diffusion and minimum inhibitory concentration evaluator (M.I.C.E; Oxoid), for their ability to determine the susceptibilities of *A. butzleri*isolates to commonly used antimicrobials.

2. MATERIALS AND METHODS

A total of seventy five *Arcobacterbutzleri* isolates from cattle (n=27), beef (n=36), dairy milk (n=9) and cattle farm environment (n=3) were isolated using the isolation method as described by Shah *et al.* (2011b). In addition to test isolates, a reference strain of *A. butzleri* (CCUG 17812) was also used as positive control. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *S. aureus* (CCUG 15915) were used as quality control (QC) organisms while testing the antimicrobial profile of *A. butzleri*.

Disc diffusion technique:

Initially, antimicrobial resistance profiles of *A. butzleri* were determined by disc diffusion method as recommended by the Clinical and Laboratory Standards

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Institute (CLSI, 2010). In brief, suspensions with 0.5 McFarland turbidity were prepared from pure culture in Mueller-Hinton broth (Oxoid, UK) by mixing the bacterial cells from fresh culture plates. Later, the cell suspension was swabbed onto Muller-Hinton plates to produce a lawn of bacterial growth and plates were allowed to dry at 37°C for 5 min before antibiotic discs were applied onto the agar. Plates were incubated aerobically at 30^0C for 48 h and zone diameters of inhibition were measured by caliper. The antibiotic discs used and breakpoints followed are given in (**Table-1)**.

Table-1 Breakpoints for the M.I.C.E and disc diffusion techniques used to profile antimicrobial susceptibility of *A. butzleri* isolates

Agent	MICE		Disc diffusion	
	Strip range(µg ml^{-1})	Breakpoint (µg ml^{-1}) S/R	Disc conc. (µg)	Zone diameter (mm) S/R
Erythromycin*	0.015-256	≤ 0.5/≥ 8	15	≥23/≤13
Ampicillin[1s]	0.015-256	≤ 8/≥ 32	10	≥17/≤13
Ciprofloxacin[1]	0.002-32	≤ 1/≥ 4	05	≥21/≤15
Cefotaxime[1]	0.002-32	≤ 1/≥ 4	30	≥21/≤13
Gentamicin[1]	0.015-256	≤ 4/≥ 16	10	≥15/≤12
Tetracycline[1]	0.015-256	≤ 4/≥ 16	30	≥19/≤14

S, susceptible; R, resistant
*MIC and zone diameter breakpoints recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (2002)
[1]MIC and zone diameter breakpoints for *Enterobacteriaceae* as recommended by the CLSI (2010)

Minimum inhibitory concentration evaluator (M.I.C.E):

Suspension of the isolates was prepared in Mueller-Hinton broth (Oxoid, UK)adjusting the turbidity equal to 0.5 McFarland standards (~$1.5x10^8$ cfu/ml). Thereafter, the suspensions were swabbed onto 150 mm diameter Mueller-Hinton agar plates supplemented with 5% sheep blood (Oxoid, UK). The agar surfaces were allowed to dry at room temperature for 10 min and a single M.I.C.E strip was applied onto each plate. Plates were incubated aerobically at 30ºC for 48 h and inhibitory concentrations (MICs) were read at the point where the elliptical zone of inhibition intersected the test strip and that concentration of the antibiotic was considered as MIC for the organism. Reading of each strip was recorded and classified as being resistant based on MIC breakpoints as recommended for *Enterobacteriaceae* (CLSI, 2010). The antibiotic strips used are given in **Table-1**.Each technique, M.I.C.E and disc diffusion was repeated twice and means were used for comparative analysis. The results of two methodologies were compared using Fischer's exact test, (two tailed).
http://www.graphpad.com/welcome.htmatP≤0.05 significance level.

3. RESULT

The prevalence of antimicrobial resistance in *A. butzleri* to six antibiotics using two techniques, M.I.C.E and disc diffusion was recorded as 89.3% and 92% for ampicillin, 22.7 and 26.7% for ciprofloxacin, 77.7 and 57.3% for erythromycin, 9.4 and 13.3% for tetracycline, 53.3 and 73.3% for cefotaxime and 22.6 and 26.7% for gentamycin, respectively**(Table-2)**.Moreover, multi-drug resistant was noticed in 16% by M.I.C.E and 12% by disc diffusion methods**(Figure-1)**.To the best of authors' knowledge, this is the first study carried out to compare two agar diffusion based antimicrobial sensitivity methods, M.I.C.E and disc diffusion for antimicrobial resistance profiling of *A. butzleri* isolates from animals and foods of animal origin.Non-significant difference (P= ≥0.05) was found in resistance profiles of 75 *A. butzleri* isolates for four of the six antimicrobials tested using M.I.C.E and disc diffusion methods **(Table-2).** However, results for erythromycin and cefotaxime were statistically significant ((P=0.0144 and P=0.0173, respectively) between two testing methods. As shown in table 2, more isolates (77. 7%) were determined as resistant with M.I.C.E compared to disc diffusion method (57.3%). Examination of the resistant populations for each antimicrobial resistant on both tests ranged from 6.7% (tetracycline) to 77.7% (ampicillin). Disc diffusion method classified higher percentage of isolates as resistant than the M.I.C.E for ampicillin (92%), ciprofloxacin (26.7%) and tetracycline (13.3%), cefotaxime (73.3%) and gentamycin (26.7%), while the M.I.C.E categorize more isolates as resistant to erythromycin (77.3%).

Table- 2. Number (%) of *A. butzleri* isolates found to be resistant to M.I.C.E and disc diffusion techniques for six antimicrobial agents tested

Antimicrobial agents tested	No. of isolates with the following classification result			No. of resistant isolates by M.I.C.E (%) (a+b)	No. of resistant isolates by disc diffusion (%) (a+c)
	a	B	c		
Ampicillin	56 (77.7)	11 (14.6)	13 (17.3)	67 (89.3)	69 (92)
Ciprofloxacin	13 (17.3)	4 (5.3)	7 (9.3)	17 (22.7)	20 (26.7)
Erythromycin	23 (30.7)	35 (46.7)	20 (26.7)	*58 (77.3)	*43 (57.3)
Tetracycline	5 (6.7)	2 (2.7)	5 (7.1)	7 (9.4)	10 (13.3)
Cefotaxime	27(36)	13 (17.3)	28 (37.3)	*40 (53.3)	*55 (73.3)
Gentamycin	9 (12)	8 (10.7)	11 (14.7)	17 (22.6)	20 (26.7)

A, Resistant To Both Tests
B, Resistant To M.I.C.E Method Only
C, Resistant to disc diffusion method only
*Denotes statistical difference between observed resistant profiles by two methodologies

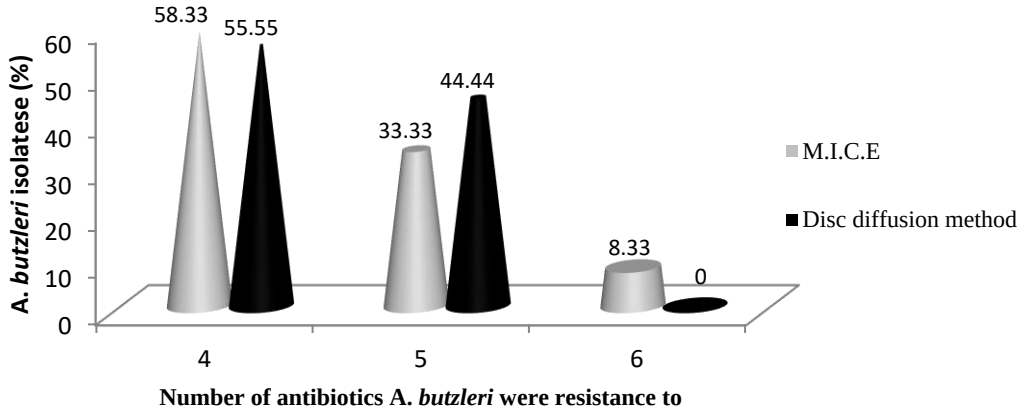

Fig. 1: Multi-drug resistant (MDR) in *A. butzleri* determined by M.I.C.E and disc diffusion techniques

4. DISCUSSION

In the present study, the highest antimicrobial resistant in *A. butzleri*was found against ampicillin (89.3%, M.I.C.E; 92%, disc diffusion) whereas *A. butzleri*showed lowest resistance to tetracycline (9.4%, M.I.C.E; 13.3%, disc diffusion). Regarding antimicrobial resistance against ampicillin, the present findings are in agreement with those published previously using various methods (Zacharow *et al.*, 2015; Unver *et al.*, 2013; Shah *et al.*, 2013; Collado and Figueras, 2011). *β-lactam*resistance in *Arcobacter* is mediated by *lrg AB*operonwhich alters the penicillin binding proteinsites (Collado and Figueras, 2011). Tetracycline was reported as the most effective in-vitro agents against *A.butzleri* isolates using dilution method (Zacharow *et al.*, 2015; Rahimi, 2014; Vandenberg *et al.*, 2006).

A. *butzleri*was found less resistant against ciprofloxacin (22.7%, M.I.C.E; 26.7%, disc diffusion) and gentamycin (22.6%, M.I.C.E; 26.7%, disc diffusion). Gentamycin and ciprofloxacin were reported as an effective antimicrobial agent against A. *butzleri* (Ferreira *et al.*, 2013; Vandenberg *et al.*, 2006). Quite high resistance (55.8%) compared to the present study, against ciprofloxacin was reported by Ferreira *et al.* (2013). The resistance to ciprofloxacinis mediated by mutation in the *Thr-85*residueof the *A. butzleri* and *A. cryaerophilusgyr A*gene (Collado and Figueras, 2011).Present results showed resistance (53.3%, M.I.C.E; 73.3%, disc diffusion) in A. *butzleri* against cefotaxime, a third generation Cephalosporin antibiotic. Cefotaximehas never been tested before against *Arcobacter* spp. Some other members of this group were tested and high resistance was noticed for cefoperazone (97.43%), cefuroximesodium (100%) and cephalothin (100%) (Atabay and Aydin, 2001). The isolates were found resistant to erythromycin at the rate of 77.7% by M.I.C.E technique and 57.3% by disc diffusion method. Similar results were reported by Zacharow *et al.* (2015). On the contrary low resistance was reported against erythromycin by Collado and Figueras (2011) and Unver *et al.* (2013) using disc diffusion tests. This difference could be due to

frequency of using this drug in the field. Unavailability of standard methods and resistance breakpoints, and frequency of antibiotic usage in the field are the major factors bringing inthe variability in the susceptibility results (Vandenberg *et al.,* 2006). Different criteria have been implicated to categorize the *Arcobacter* as MDR. Some authors categorize *Arcobacter* as MDR if it is resistant to three or more drugs (Son *et al.,* 2007) while others follow the criterion of four or more agents (Harras *et al.,* 1998).A total of 16% by M.I.C.E and 12% by disc diffusion methods isolates were found as multidrug resistant bacteria (resistant to four or more antibiotics). Multidrug resistance in *Arcobacter* has also been reported by some other research groups at varying levels using different methodologies. By using micro dilution method, Son *et al.* (2007) reported 24% isolates as resistant to three antimicrobials. Similarly, multidrug resistance to ampicillin, erythromycin and nalidixic acid was observed in 6.2% of *A. butzleri* strains (Vandenberg *et al.,* 2006). Harrass *et al.* (1998) reported 15.73% MDR *A. butzleri*isolates. Bacteria use multiple strategies to overcome static or lytic actions of antimicrobial agents. Many antimicrobial resistance determinants such as plasmids, transposons, multi drug efflux pump, and integrons, have been involved in the evolution and dissemination of multidrug resistance in bacteria (Kinana *et al.,* 2009; Xu *et al.,* 2009; Labbate *et al.,* 2008; Liu *et al.,* 2008).The differences in the results of the present study from those of others are possibly due to the method applied, source of isolates and cut of value to describe MDR. No significant differences ($p= \geq 0.05$) were found in resistance profiles of *A. butzleri* isolates for four of the six antimicrobials tested using either of the methods however discrepancy between the two methods was recorded for erythromycin and cefotaxime. More isolates were found resistant against cefotaxime using disc diffusion method probably due to high concentration of the disc or low percentage of isolates were resistant to cefotaxime using M.I.C.E occurred due to inequitable concentration increment for the drug. More studies are required in order to harmonize the results of erythromycin and cefotaxime with M.I.C.E and disc diffusion methods. Some other comparative studies on antimicrobial profiling methodologies have also been carried out. McGill *et al.* (2009) profiled antibiotic resistance patterns of 75 *Campylobacter* isolates of food and human clinical isolates using disc diffusion and E-test methods and found that both methods showed non-significant results for four antibiotics tested (ciprofloxacin,nalidixic acid, chloramphenicol, and tetracycline) but the results were significantly different for two antibiotics, ampicillin and erythromycin. Similarly, Luangtongkum *et al.* (2007) compared disc diffusion method to a standardized agar dilution method of McDermott *et al.* (2004) for antimicrobial sensitivity testing in 686 *Campylobacter* isolates from poultry and reported non-significant differences in the results of two methods for ciprofloxacin, erythromycin, tetracycline and nalidixic acid. Erfani *et al.* (2011) compared E-test versus disc diffusion methods to determine the multi-drug resistance in *Escherichia coli* and generally concluded that the E-testwas more accurate and superior to disc diffusion in detecting multi-drug resistance. Emergence of antimicrobial resistance against commonly used antibiotics is an alarming signal to animals and public health. Tetracycline can be used as a first-line drug to treat infection caused by *A. butzleri*. The results from two antimicrobial susceptibility testing showed correlation between two methods for ampicillin, ciprofloxacin, tetracycline and gentamycin thus either of the methods may be used for in-vitro antibiotic sensitivity testing of *A. butzleri*. Each method has own advantages such as disc diffusion is cost effective whereas, M.I.C.E is quite expensive but it has advantages over the disc diffusion by serving as a guide for selection of proper drug and its therapeutic concentration that is to be administered to give an effective treatment so as to avoid the emergence of antimicrobial resistance. From various studies, it is evident that further validation of these methods is needed and suitable internationally accepted breakpoints are to be established through multi-laboratory quality control studies.

REFERENCES:

Atabay H. I. and F. Aydin, (2001). Susceptibility of *A. butzleri* isolates to 23 antimicrobial agents. Letter Applied Microbiology, 33: 430-433.

Clinical and Laboratory Standards Institute, (2010). Performance standards for antimicrobial susceptibility testing. Twelfth Informational Supplement M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute, 30, No. 1.545-550.

Collado, L. and M. J. Figueras, (2011). Taxonomy, epidemiology, and clinical relevance of the genus *Arcobacter.* Clinical Microbiology Review, 24(1): 174–192.

Douidah, L., L. De Zutter, F. Van Nieuwerburgh, D. Deforce, H. Ingmer, O. Vandenberg,A.M. Van den Abeele and K. Houf, (2014). Presence and Analysis of Plasmids in Human and Animal Associated *Arcobacter* Species. PLoS ONE 9(1): 485-487.

Erfani Y, A. Rasti, A. Mirsalehian, S. M. Mirafshar and B. Ownegh, (2011). E-test versus disc diffusion method in determining multi-drug resistant strains of *E. coli* in urinary tract infection. African Journal of Microbiological Research, 5(6): 608-611.

Ferreira S., M. J. Fraqueza, F. C. Domingues and M. Oleastro, (2013). Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacterbutzleri* isolated from poultry and environment from a Portuguese slaughterhouse. International Journal of Food Microbiology, 162(1): 82-88.

Harrass, B., S. Schwarz, and S. Wenzel, (1998). Identification and characterization of *Arcobacter* isolates from broilers by biochemical tests, antimicrobial resistance patterns and plasmid analysis. Zentralblatt fur Veterinarmedizin Reih, 45: 87-94.

Hsu, T. T. D and J. Lee, (2015). Global distribution and prevalence of *Arcobacter* in food and water. Zoonosis and Public Health 62: 579-589.

ICMSF, (2002). Microorganisms in food? In Microbiological Testing in Food Safety Management. Kluwer/Plenum, Dordrecht/New York, Pp. 171

Kinana, A. D., V. Ricci and L. J. V. Piddock, (2009). Contribution of efflux to antibiotic resistance in *Campylobacter* isolated from poultry in Senegal. Journal of Antimicrobial and Chemotherapy Research Letter, 650-652.

Labbate, M., P. R. Chowdhury and H. W. Stokes, (2008). A class 1 integron present in a human commensal has a hybrid transposition module compared to *Tn402*: Evidence of interaction with mobile DNA from natural environments. Journal of Bacteriology, 190 (15): 5318–5327.

Liu, Z. Q., P. Y. Zheng and P. C. Yang, (2008). Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance. World Journal of Gastroenterology, 14(33): 5217-5222.

Luangtongkum T., T. Y. Morishita, A. B. El-Tayeb, A. J. Ison andQ. Zhang, (2007). Comparison of antimicrobial susceptibility testing of *Campylobacter* spp. by the agar dilution and the agar disc diffusion methods. Journal of Clinical Microbiology 45: 590-594.

McDermott, P. F., S. M. Bodeis, F. M. Aarestrup, S. Brown, M. Traczewski, P. Fedorka-Cry, M. Wallace, C. Critchley, C. Thornsberry, (2004.) Development of a standardized susceptibility test for *Campylobacter* with quality control ranges for ciprofloxacin, doxycline, erythromycin, gentamycin, and meropenem. Microbial Drug Resistant, 10: 124-131.

McGill K., L. Kelly, R. H. Madden, L. Moran, C. Carroll, A. O'Leary, J. E. Moore, E. McNamara, S. Fanning and P. Whyte, (2009). Comparison of disc diffusion and epsilometer (E-test) testing techniques to determine antimicrobial susceptibility of *Campylobacter* isolates of food and human clinical origin. Journal of Microbiological Methods 79: 238-241.

Rahimi, E., (2014). Prevalence and antimicrobial resistance of *Arcobacter* species isolated from poultry meat in Iran. Broiler Poultry Science, 55(2):174-80.

Shah, A. H., A. A. Saleha, Z. Zunita and M. Murugaiyah, (2011a) *Arcobacter*-An emerging threat to animals and animal origin food products? Trends in Food Science and Technology, 22: 225-236.

Shah, A. H., M. Murugaiyah and Z. Zunita, (2011b). Assessment of sensitivity, specificity and species discriminatory power of four culture-based isolation methods of *Arcobacter* spp. African Journal of Microbiological Research, 5: 3753–3759.

Shah, A. H., A. A. Saleha, Z. Zunita, M. Murugaiyah, A. B. Aliyu and N. Jafri, *(*2013). Prevalence, distribution and antibiotic resistance of emergent *Arcobacter* spp. from clinically healthy cattle and goats. Trans Emerging Diseases60 (1):9-16.

Son, I., M. D. Englen, M. E. Berrang, P. J. Fedorka-Cray and M. A. Harrison, (2007). Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. International Journal of Antimicrobial Agents, 29 (4): 451-455.

Unver, A., H. I. Atabay, M. Sahin and O. Celebi, (2013). Antimicrobial susceptibilities of various *Arcobacter* species. Turkish Journal of Medicine Science 43:548-552.

Van den A., A. M., D. Vogelaers, J. Van Hende and K. Houf, (2014). Prevalence of *Arcobacter*species among humans, Belgium, 2008–2013. Emerging Infectious Diseases, 20(10):1746–9.

Vandenberg, O., A. Dediste, K. Houf, S. Ibekwem, N. Douat, G. Zissis, J.P. Butzler, and P. Vandamme, (2004). *Arcobacter* species in humans. Emerging Infectious Diseases, 10(10):1863–7.

Vandenberg, O., K. Houf, N. Douat, L. Vlaes, P. Retore, B. Jean-Paul and A. Dediste, (2006). Antimicrobial susceptibility of clinical isolates of non-*jejuni*/*coli Campylobacter* and *Arcobacter* from Belgium. Journal of Antimicrobial and Chemotherapy, 57: 908–913.

Xu, H., Z. Su, S. Wang, X. Dai, J. Chen, F. Kong, Y. Li, S. Peng, Q. Shao, L. Lu andT. Ezaki, (2009). Four novel resistance integron gene-cassette occurrences in bacterial isolates from Zhenjiang, China. Current Microbiology, 59: 113–117.

Zacharow, I., J. Bystron, E. Wałecka-Zacharska, M. Podkowik and J. Bania, (2015). Prevalence and antimicrobial resistance of *Arcobacterbutzleri* and *Arcobactercryaerophilus* isolates from retail meat in Lower Silesia region, Poland. Poland Journal of Veterinary Sciences, 18(1):63-69.