Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

SHORT COMMUNICATION

Clonal Expansion of Sulfonamide Resistant *Escherichia coli* Isolates Recovered from Diarrheic Calves

Muhammad Adnan[1], Hamayun Khan[2], Jam Kashif,[1] Shakoor Ahmad[2], Ali Gohar[3], Amjad Ali[4], Muhammad Atif Khan[1], Said Sajjad Ali Shah[2], Mohammad Farooque Hassan[7], Muhammad Irshad[5], Nazir Ahmad Khan[6] and Sadeeq ur Rahman[3*]

[1]Department of Pharmacology, Sindh Agriculture University Tando Jam; [2]Department of Animal Health, The University of Agriculture Peshawar; [3]College of Veterinary Sciences and AH, Abdul Wali Khan University, Mardan; [4]Centre for Human Genetics, Hazara, University, Mansehra- Pakistan; [5]Department of Preventive Dentistry, Aljouf University, Kingdom of Saudi Arabia; [6]Department of Nutrition, The University of Agriculture, Peshawar; [7]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Sakrand Sindh, Pakistan
*Corresponding author: sadeeq@awkum.edu.pk

ARTICLE HISTORY (16-278)

Received: October 29, 2016
Revised: January 20, 2017
Accepted: February 03, 2017
Published online: March 07, 2017

Key words:
Diarrhea
E. coli
Minimum inhibitory concentration
Sul genes
Sulfadimidine resistance

ABSTRACT

A total of 30 *Escherichia coli* isolates from 50 random samples of diarrheic calves were screened against resistance to sulfonamides. Minimum inhibitory concentration (MIC) by microdilution method indicated that 25/30 (83.3%) were found resistant to sulfadimidine, 27/30 (90%) were resistant to sulfadiazine, while 22/30 (73.3%) were found resistant to sulfamethaxole. These phenotypically sulfonamide resistant isolates were then probed for the presence of *Sul* genes (*Sul*-1-3) by polymerase chain reaction (PCR) using specific primers. PCR results revealed that 23/25, 25/27 and 20/22 isolates carried *Sul*-1 gene, respectively. Interestingly, all these isolates were found negative for the presence of *Sul*-2 and *Sul*-3 genes. Of note, no *Sul* genes could be verified in 02 phenotypically resistant isolates. The abundance of *Sul1* gene and absence of *Sul*-2 and *Sul*-3 genes indicating a clonal expansion of sulfonamide resistant *E. coli* that might be linked to excessive abuse of sulfonamides in animals.

To Cite This Article: Adnan M, Khan H, Kashif J, Ahmad S, Gohar A, Ali A, Khan MA, Shah SSA, Hassan MF, IrshadM, Khan NA and Rahman SU, 2017. Clonal expansion of sulfonamide resistant *Escherichia coli isolates* recovered from diarrheic calves. Pak Vet J, 37(2): 230-232.

INTRODUCTION

Sulfonamide has been used extensively to treat bacterial and protozoal infection since many decades. In a veterinary context, sulfonamides are frequently used alone or in combination with trimethorpim or other antibiotics both for prophylactic and treatment of diarrhea in intensive animal care units. Sulfonamides, structural analogs of p-amino benzoic acid, compete with p-amino benzoic acid for binding to dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid synthesis cycle; thereby inhibiting synthesis of dihydrofolic acid (Sköld, 2000) and thus inhibit the growth of bacteria.

Resistance to sulfonamides in *E. coli* can arise either due to chromosomal mutations of the DHPS (*folP*) gene or more frequently from acquisition of an alternative DHPS genes, like *sul1*, *sul2* (Sköld, 2000) and *sul3* gene. Despite of maintaining more than 50% homology at the DNA level, these sulfonamide resistance genes (*sul1-sul3*) have been clearly classified separately from the chromosomal mutated DHPS gene. Presence of the *sul* genes in the transferable mobile elements such as plasmids is linked to the most of the current sulfonamide resistance epidemics around the globe. Unfortunately, in Pakistan, extent of antibiotic resistance, and in particular, resistance to sulfonamides is under reported.

Sulfonamides, particularly, combination of trimethoprims and sulfonamide is widely used to treat diarrhea in farm animals. Thus the persistent use of these drugs would provide selective pressure to pathogenic *E. coli* to develop resistant against these drugs. Pathogenic *E. coli* resistant to sulfonamides are excreted in the feces, contaminating environment and water that speeds up dissemination. Due to lack of data, it is hard to document the current level, types and prevalence of bacterial resistance in different regions of Pakistan. The current study was thus designed to determine the level and nature of sulfonamide resistance in *E. coli* recovered from diarrheic calves.

MATERIALS AND METHODS

Sample collection and processing: The study was conducted between March-December-2015. A total 50 rectal swab samples were collected from diarrheic calves (age 1week to 1 year) in random from five different cattle farms located in Hyderabad and processed by following guidelines issued by the clinical and standard laboratory institute (CLSI) (CLSI, 2014). Standard procedures for *E. coli* isolation were followed.

Antimicrobial susceptibility testing: Minimal inhibitory concentrations (MICs) of *E. coli* isolates were determined using the standard broth doubling micro broth dilution method on Muller–Hinton medium as described by CLSI (CLSI, 2014). Antimicrobials used in this study were sulfadimidine, sulfadiazine and sulfamethaxole. Plates were incubated at 37ºC overnight and bacterial growth was interpreted by turbidity and breakpoints were recorded following CLSI guidelines. Reference strain of *E. coli* ATCC 25922 was used alongside the isolates for susceptibility testing. All materials were purchased from Oxoid (Thermo Fisher Scientific Oxoid Ltd., United Kingdom).

Genotypic confirmation and characterization: DNA from phenotypically sulfonamide resistant *E. coli* isolates was extracted using plasmid DNA extraction kits from Takara (TaKaRa FastPure™ DNA, Osaka, Japan) following manufacturer's instruction. Primers used in the study are listed in Table 1. Primers were synthesized by Invitrogen. PCR products were analyzed through electrophoresis using 1% agarose gel. The molecular weights of amplified DNA bands were compared a known molecular weight DNA marker (Thermoscientific Fischer).

Table-1: Specific primers of *Sul* genes were used for PCR

Gene	Primer	Sequence (5` to 3`)	Annealing temp (°C)	Ref.
Sul-1	*Sul*1F	ACTTCAACGATGAGAGCCGG	55-60	(Kerrn *et al*., 2002)
	*Sul*1R	GCGATCGAAATGCTGCGAGT		
Sul-2	*Sul*2F	TCGTCAACATAACCTCGGACAG	55-60	(Singha *et al*., 2015)
	*Sul*2R	GTTGCGTTTGATACCGGCAC		
Sul-3	*Sul*3F	GAGCAAGATTTTTGGAATCG	55-60	(Bean *et al*., 2009)
	*Sul*3R	CATCTGCAGCTAACCTAGGGC		

RESULTS AND DISCUSSION

Our results indicated that 30 of the 50 samples were declared positive for the presence of *E. coli*. These isolates were further tested for sulfonamide resistance. In line with our findings, a prospective study involving investigation of causes of early calf mortality established that *E. coli* was the most common isolated pathogens (Osman *et al*., 2013). At the farm level, calves infected with diarrhea are mainly treated with sulfonamide drugs. Therefore, we speculated that the frequent exposure to sulfonamides might have triggered selective emergence of resistant. Indeed, our results indicated that 25 isolates displayed resistance to sulfadimidine, 27 isolates showed resistant to sulfadiazine and 22 were found resistant to sulfamethaxole. Interestingly, 12 isolates showed resistance to all of the tested sulfonamides (sulfadimidine, sulfadiazine and sulfamethaxole; MIC>512 µg/ml). At least, 10 isolates indicated a relatively higher MIC (128 µg/ml) against at least one of the three sulfonamides tested. Sulfonamide resistance has been reported from all over the world including United States and European Union. However, incidence of resistance level is comparatively higher in Asia where as many as 92% of clinical *E. coli* isolates have been reported resistant to sulfonamides (Srinivasan *et al*., 2007). This higher resistance pattern is most probably linked to the frequent usage of sulfonamide drugs for treatment purposes in practices (Sköld, 2000).

Sulfonamide resistance is attributed to one of the three (*Sul*1-3) genes that can be encoded in the chromosome or most oftenly present on the mobile elements such as plasmid. We minipreped the plasmid and then applied PCR to target the *Sul* genes located in the plasmid only. The PCR results revealed that 23/25, 25/27 and 20/22 isolates carried *Sul*-1 gene only, however no *Sul* gene was present in 02 isolates, which were phenotypically resistant to Sulfonamides (Fig.1), whereas, no *Sul*-2 or *Sul*-3 genes were present (results not shown) as revealed by others (Chung *et al*., 2015). Our inability of detection of *sul* genes in the otherwise phenotypically sulfonamide resistance strains more likely indicates the absence of these genes in the plasmid. Other mechanisms of sulfonamide do exist, such as, mutation in the DHPS gene or other broader biochemical mechanisms (Hammerum *et al*., 2006). It would be interesting to investigate these isolates for alternative modes of sulfonamide resistance. Of note, as expected, the 3/30 sulfonamide susceptible isolates also did not reveal any PCR product. Our results indicated a higher prevalence of *Sul1* genes among the phenotypically sulfonamide resistant isolates as reported earlier (Vinué *et al*., 2010). None of our isolates carry any other *sul* gene (*sul*2 *or sul*3), albeit, a number of reports indicated that *Sul2*- and *Sul3*- genes have also been found conferring resistant to sulfonamide (Hammerum *et al*., 2006). *Sul1* gene has often reported associated with integron 1 in the 3' conserved segment (3'-CS), and demonstrated rare sequence variations. Sequence analysis of one of the candidate *sul1* gene of our recent published work against 100 known *sul1* genes at the integrall data base [http://integrall.bio.ua.pt/?search#] showed a 100% identity with E value 0 (Fig 1b). Data regarding antibiotic resistance is rare in Pakistan; however, the available data indicated higher prevalence of sulfonamide resistant pathogens (Khan *et al*., 2013). Interestingly, in our case, *Sul*1 gene was carrying in the plasmid revealing a potential threat of fast dissemination as plasmids could be horizontally exchanged between similar species. Our results corroborate with earlier findings of the presence of *Sul* genes on the mobile elements such as plasmid, which represents the most prevalent cases globally.

Overall, we report on the widespread dissemination of *Sul1* genes that is involved conferring resistant to sulfonamide in pathogenic *E coli* clinical strains isolated from diarrheic calves in Sindh province of Pakistan.

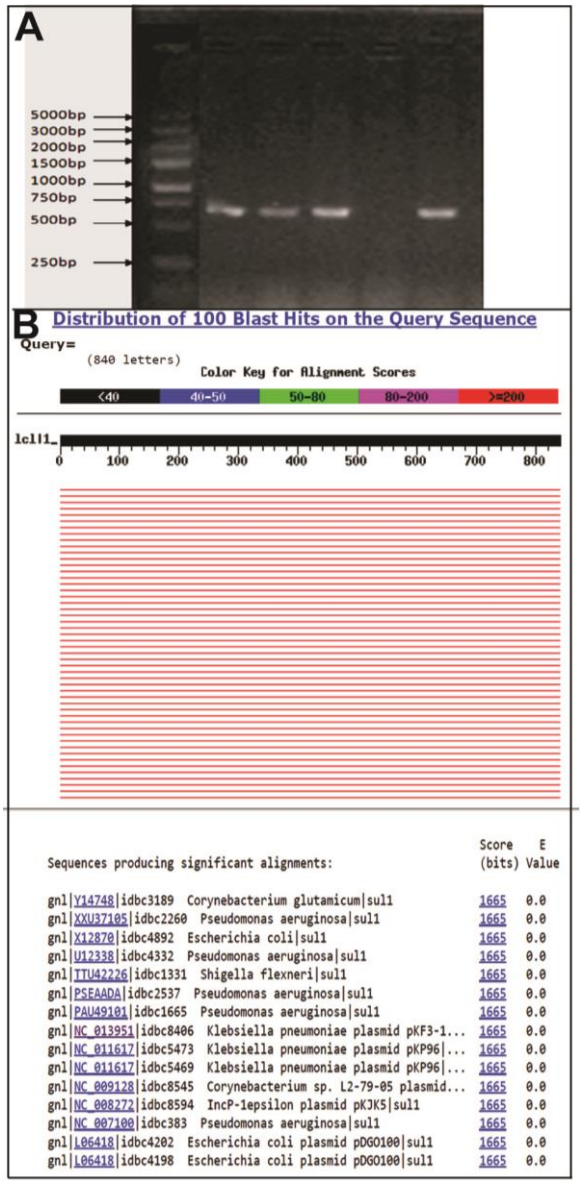

Fig. 1: (a) Above picture indicates PCR based detection of *Sul1* gene from the plasmid minipreps of *E. coli* isolates from calves that were infected with diarrhea. Representative samples are shown. S-1, S-3, S-4 and S-6 are the isolates that were phenotypically resistant to sulfonamides, while –ve was the susceptible strain that was found negative for the PCR amplicon (b) blast analysis of one of *sul1* gene against 100 known *sul1* gene in the integrall reprotoire. Color key for alignment is on the top and E values (representative) are given underneath.

Acknowledgments: The authors acknowledge the cooperation of the Sindh Poultry Vaccine Centre, Karachi and the University of Agriculture, Peshawar for support in providing lab space to perform the work.

Authors contribution: MA, JK, AG, AA and MFH helped in sample collection and isolation of *E. coli* isolation. MA, MAK, SSS and MI provided reagents for PCR and helped in performing PCR. SUR, NAK, HK and SA conceived and designed the project and drafted the manuscript. All the authors have revised the manuscript and approved the final version.

REFERENCES

Bean DC, Livermore DM and Hall LM, 2009. Plasmids imparting sulfonamide resistance in *Escherichia coli*: implications for persistence. Antimicrob Agents Chemother 53:1088-93.

Chung HS, Kim K, Hong SS, *et al.,* 2015. The sul1 gene in Stenotrophomonas maltophilia with high-level resistance to trimethoprim/sulfamethoxazole. Ann Lab Med 35:246-9.

Hammerum AM, Sandvang D, Andersen, SR, *et al.*, 2006. Detection of sul1, sul2 and sul3 in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. Int J Food Microbiol 106:235-7.

CLSI, 2014. Performance Standards for Antimicrobial Susceptibility Testing. Wayne, PA: Clinical and Laboratory Standard Institute. CLSI document M100-S124.

Kerrn M, Klemmensen T, Frimodt-Møller N, *et al.*,, 2002. Susceptibility of Danish Escherichia coli strains isolated from urinary tract infections and bacteraemia, and distribution of sul genes conferring sulphonamide resistance. J Antimicrob Chemoth 50:513-6.

Khan GA, Berglund B, Khan KM, *et al.*, 2013. Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities–a study in Pakistan. Plos One 8:62712.

Osman K, Mustafa A, Elhariri M, *et al*., 2013. The Distribution of *Escherichia coli* serovars, virulence genes, gene association and combinations and virulence genes encoding serotypes in pathogenic *E. coli* recovered from diarrhoeic calves, sheep and goat. Transbound Emerg Dis 60:69-78.

Singha P, Maurya AP, Dhar D, *et al.*, 2015. Sulphonamide resistance in clinical isolates of *Escherichia coli* and their association with class I integron: A study from India. AC Microb 6:1-5.

Sköld O, 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist Update 3:155-60.

Srinivasan V, Gillespie BE, Lewis MJ, *et al.,* 2007. Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. Vet Microbiol 124:319-28.

Vinué L, Sáenz Y, Rojo-Bezares B, *et al.* 2010. Genetic environment of sul genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. Int J Antimicrob Ag 35:492-6.