Journal of Nepal Agricultural Research Council
Vol.9: 104-113, December 2023
ISSN: 2392-4535 (Print), 2392-4543 (Online)
DOI: https://doi.org/10.3126/jnarc.v9i1.61601

Influence of Incubation Condition on the Antibiotic Susceptibility of Salmonella

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Received: Oct. 8, 2023, Revised: Agutust.12, 2023, Accepted: Sept. 2, 2023, Published : December 27, 2023.

The authors declare that there is no conflict of interest.

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ABSTRACT

Bacteria experience changes in physiology and growth patterns in aerobic laboratory culture and the anaerobic gastrointestinal tract of the host. Our study hypothesizes that this physiological difference influences the antibiotic susceptibility/resistance pattern of *Salmonella* in aerobic and anaerobic conditions. The susceptibility and resistance percentage was determined using the diameter of the zone of inhibition for ten major classes of antibiotic drugs with a total of sixteen molecules. Data were analyzed in GraphPad Prism vs 8 using a two-way ANOVA analysis for the six antibiotics selected based on the greatest differences in the zone of inhibition. The highest observed resistance levels were against nalidixic acid (63.8%/48.6%) and tetracycline (65.2%/58.6%), followed by trimethoprim/ sulphamethoxazole (52.2%/50%), cephalexin (50.7%/52.9%), ciprofloxacin (45.7%/45.7%), amoxicillin (43.5%/47.1%), ampicillin (37.7%/45.7%) and chloramphenicol (36.2%/37.1%) in aerobic/anaerobic condition. The variation contributed by the type of antibiotic on the degree of resistance was 72.73% (P=0.0303) and the variation contributed by the incubation condition was 16.09% (P=0.0437), both of which are statistically significant. The findings of our study demonstrate that the susceptibility/resistance of non-typhoidal Salmonella varies in aerobic and anaerobic incubation. Hence, pharmacodynamics models aiming to evaluate the impact of antimicrobial use in enteric bacteria such as *Salmonella* of the treated host should utilize measurements of bacterial susceptibility that are obtained anaerobically (as well as aerobically) to achieve effective antimicrobial treatment and control non-typhoidal salmonella infections.

Keywords: Incubation Condition, Antibiotic Agent, Resistance, Salmonella

सारांश

जिवाणुहरुको फिजियोलोजी र वृद्धि ढाँचामा प्रयोगशालामा हुने एरोविक वातवरण र होस्टको पाचन प्रणाली खासगरी अन्द्रामा हुने एनएरोविक वातावरणले फरक प्रकारको असर गर्न सक्छ । हाम्रो अध्ययनले परिकल्पना गर्दछ कि यो एरोबिक र एनारोबिक अवस्थाहरूको भिन्नताले साल्मोनेलाको एन्टिबायोटिक संवेदनशीलता/प्रतिरोध ढाँचालाई पनि प्रभाव पार्छ । संवेदनशीलता तथा प्रतिरोध अध्ययन, दश प्रमुख समूहका कुल सोह्र प्रकारका एन्टिबायोटिक औषधिको लागी निषेध क्षेत्रको व्यासको उपयोग गरी निर्धारण गरिएको, अवरोधको क्षेत्रमा सबैभन्दा ठूलो भिन्नताको आधारमा चयन गरिएका छ वटा एन्टिबायोटिकहरूका लागि डेटाको विश्लेषण ग्राफप्याड प्रिज्म मा दुई-तर्फी ANOVA प्रयोग गरी गरिएको थियो । एरोबिक/एनारोबिक अवस्थामा उच्चतम प्रतिरोध स्तरहरू नालिडिक्सिक एसिड (६३.८%/४८.६%) र टेट्रासाइक्लिन (६५.२%/५८.६%), त्यसपछि ट्राइमेथोप्रिम/सल्फामेथोक्साजोल (५२.२%/५०%), सेफलेक्सिन (५०.७%/५२.९%), सिफालेक्सिन (५०.७%/५२.९%), सिप्रोफ्लोक्सासिन ४५.७%/४५.७%), एमोक्सिसिलिन (४३.५%/४७.१%), एम्पिसिलिन (३७.७%/४५.७%) र क्लोराम्फेनिकोल (३६.२%/३७.१%) अवलोकन गरिएको थियो। प्रतिरोधको डिग्रीमा एन्टिबायोटिकको

प्रकारले योगदान गरेको भिन्नता ७२.७३%, P=0.0303, थियो भने इन्क्युवेशन अवस्थाले योगदान गरेको भिन्नता १६.०९%, P=0.0437, थियो, जुन सांख्यिकीय रूपमा महत्त्वपूर्ण छन् । हाम्रो अध्ययनको निष्कर्षले देखाउँछ कि गैर-टाइफाइडल साल्मोनेलाको संवेदनशीलता/प्रतिरोध एरोबिक र एनारोबिक इन्क्युवेशनमा भिन्न हुन्छ । तसर्थ, साल्मोनेला जस्ता इन्टेरिक जिवाणुमा एन्टिमाइक्रोबियल प्रयोगको प्रभावको मूल्याङ्कन गर्नेका हेतु प्रयोग गरिने फार्माकोडाइनामिक्स मोडेलहरू र प्रभावकारी उपचारका लागी एनारोबिकका साथसाथै एरोबिक रूपमा प्राप्त गरिएको ब्याक्टेरियाको संवेदनशीलताको मापन प्रयोग गर्नु पर्छ ।

INTRODUCTION

Salmonella, of the family Enterobacteriaceae, is an important zoonotic pathogen whose transmission is primarily via the feco-oral route. It causes typhoid fever, gastroenteritis, septicemia, and even death in infected humans (Zhai et al 2014). It is reported that approximately 90% of human salmonellosis results from ingestion of contaminated food products originating from poultry and other animals (Andino and Hanning 2015). Poultry meat and egg are allegedly the most common source of human infection as poultry birds can be asymptomatic carriers and the organism can persist along with the processing (e.g. primary production on-farm, slaughter operations, equipment, meat handlers, and retail meat) with efficient transmission and rapid spread (Antunes et al 2016). Salmonella infecting poultry are divided into two groups namely host-specific (typhoidal) and broad host range (non-typhoidal) groups (Crump et al 2015). Non-typhoid salmonella does not cause high mortality or interfere performance of the bird but is responsible for human infection through the consumption of contaminated poultry products (Foley et al 2011). The predominant non-typhoid Salmonella serovars associated with poultry and human infections are *S. enterica* serovar Enteritidis and *S.* Heidelberg, *S*. Kentucky, and *S*. Typhimurium (Foley et al 2011, Zhu et al 2015).

The extensive abuse of antibiotic agents has led to the emergence and spread of antibiotic-resistant salmonellae (Sengupta et al 2014). The disease has a negative economic impact worldwide, due to food recalls, surveillance and inspection of human infections, and treatment and prevention. Similarly, the emergence of antibiotic-resistant strains leading to therapeutic failures has also become an important problem in veterinary medicine and public health (Lathers 2001). Judicious use of an appropriate and effective antibiotic agent is the only measure to vie anti-microbial resistant (AMR) strains of enteric bacteria. Antibiotic susceptibility test (AST) is a tool to clinically identify the appropriate antimicrobial agent that can be used therapeutically. The test is generally performed *in vitro* aerobic laboratory culture while the enteric pathogen like *Salmonella* dwells in the anaerobic gut environment of the host. Bactericidal antibiotics kill organisms in their growing phase whereas bacteriostatic targets the bacterial function or physiology. The antimicrobial agents depend upon the physiology and growth rate of the organism for their action which is further determined by the environment (DeMars et al 2016). Hence, AST *in vitro* aerobic laboratory culture may not truly and accurately mimic the pharmaco-dynamic models of the pathogen within the host environment. The objective of the study was to evaluate and compare the antibiotic susceptibility of the isolates in aerobic and anaerobic conditions for multiple classes of antibiotics such as aminoglycosides, amphenicol, ß-lactams, cephalosporins, tetracycline, sulphonamides, macrolides, fluoroquinolones, polymyxin, and aminocoumarin.

MATERIALS AND METHODS

Location

The laboratory work of the research study was conducted for four months starting from 25 Feb 2020 in National Animal Health Research Centre under Nepal Agricultural Research Centre situated in Bagmati province, Lalitpur-15, Khumaltar, at latitude 27.650667˚N and longitude 85.322361˚E.

Bacterial isolates

In this study, a total of 100 random samples i.e. organism repository at -80˚C deep freezer of National Animal Health Research Centre (NAHRC) were selected. The samples for pathogen isolation were

collected from poultry litters, faeces and clinical cases of poultry (for example liver, heart, spleen and trachea). The biological samples were initially incubated at 37˚C for 20 hours in Buffer Peptone Water for pre-enrichment. The broth was then cultured in Salmonella Shigella agar and XLD agar. Typical pale colonies with black centres growing on these agar plates were stained using Gram stain and biochemical tests. SIM medium was used for the determination of hydrogen sulphide production, indole formation and motility of the pathogen. Motile, H_2S positive but indole negative, gram-negative colonies were transferred to Luria Bertani broth for overnight culture. DNA was extracted from the culture inoculated in LB broth, with the help of QIAamp DNA Kit (Qiagen) followed by end-point PCR for the *invA* gene, using TaqPCR mastermix kit and gel electrophoresis for bacteria identification. The PCR procedure entailed primers 139-1421 for detecting *Salmonella.* The procedure was performed with an initial denaturation step of 95 °C for 5 min, 35 cycles of 95 °C for the 30s, 60 °C for 30s, 72 °C for 45s and a final extension step of 72 °C for 10 min with a total time of two hours and ten minutes for completion. The PCR product was visualized under ultraviolet light after a run on 1.5% agarose gel in 1×TAE buffer [40 mM Tris-acetate, pH 8.0, 1 mM EDTA]. The antibiotic susceptibility test (AST) was performed for the PCR-positive samples using the standard protocols by Kirby-Bauer disc diffusion method in aerobic and anaerobic conditions. The anaerobic condition was maintained using a gas pack in a gas jar system (Burt and Phillips 1997).

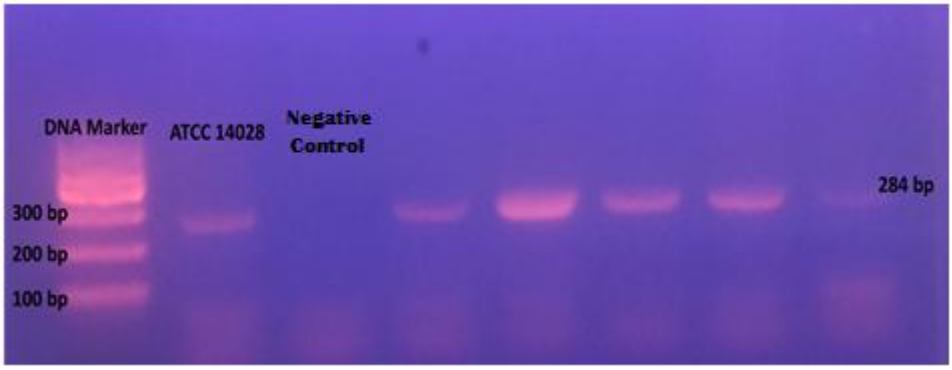

Figure 1: Representative image of the gel electrophoresis for the identification of Salmonella using the invA gene. The presence of bands at 284 bp is confirmatory for Salmonella; compare it with the presence of a band of the same size of the control strain ATCC 14028 on Lane 2, negative control on Lane 3 and DNA marker on Lane 1, starting from left.

Antibiotics

For each bacterial isolate, the zone of inhibition was interpreted as per European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2019) for the following antibiotics taken as representative of their classes- gentamicin (GEN, 10 mcg), streptomycin (STR, 10 mcg) for aminoglycosides, chloramphenicol (CHL, 30 mcg) for amphenicols, novobiocin (NOV, 30 mcg) for aminocoumarins, ampicillin (AMP, 10 mcg), amoxicillin (AMX, 10 mcg), imipenem (IMP, 10 mcg) for ß-lactams, cefalexin (LEX, 30 mcg), cefoxitin (FOX, 30 mcg), ceftriaxone (CRO, 30 mcg) for cephalosporins, azithromycin (AZM,10 mcg) for azalides, colistin (COL,10 mcg) for polymyxins, ciprofloxacin (CIP, 5 mcg), nalidixic acid (NAL, 30 mcg) for quinolones, cotrimoxazole (SXT, 25 mcg) for sulpha-trimethoprim, and tetracycline (TCY, 30 mcg) for tetracyclines. The antibiotic discs used in the study were manufactured by HiMedia, Mumbai India. These antibiotics were selected based on the evidence of usage by poultry farmers as well as their commercial availability at a reasonable price in the local market.

Antibiotic susceptibilities of bacterial isolates

For the antibiotic susceptibility test, the bacterial strains identified positively as Salmonella using the PCR were inoculated into Muller Hinton broth and incubated at 37˚C for 4 hours. The turbidity of bacterial suspension was compared with 0.5 McFarland standards (0.05ml of 1.175% barium chloride dehydrate with 9.95ml of 1% sulphuric acid). The suspensions with turbidity matching that of 0.5 McFarland were plated onto the Muller Hinton agar by lawn culture technique using a glass spreader. The antibiotic discs were then carefully placed on the plate using a disc dispenser and sterile forceps. The anaerobic condition was maintained by using an anaerobic jar. The plates were incubated at 37˚C for 18-24 hours after which the diameter of the zone of inhibition was measured with the help of a manual vernier calliper.

Statistical analysis

The data obtained from the test were entered in WHONET vs. 2020. The descriptive and graphical analyses were performed in WHONET for all sixteen antibiotic drugs used in the study. Antibiotics with the highest differences in the zone of inhibition between the aerobic and anaerobic environment were selected for two-way ANOVA analysis in Graph Pad Prism vs 8.

RESULTS

Out of 100 samples from the NAHRC organism bank, 38 samples were positive for *Salmonella* whereas all 32 clinical samples collected from OPD cases were positive by PCR test. *Salmonella* Typhimurium, ATCC 14028 (quality control commercially prepared microbiological proved ATCC 14028 was used as a positive control during the assay (**Figure** 1). Antibiotic Sensitivity Test (AST) by Kirby-Bauer disc diffusion method in aerobic and anaerobic conditions was performed on these 70 isolates confirmed to be *Salmonella* spp. The zone of inhibition was interpreted as per EUCAST guidelines into the resistant, intermediate and susceptible strains. The overall resistance and susceptibility pattern of the *Salmonella* isolates in aerobic and anaerobic conditions for the different sixteen antimicrobial drugs is represented in **Table** 1. The intermediate strains were merged with the resistance ones as antibiotics labelled intermediate are generally not recommended for use in the clinical setting (Jiang et al 2019).

Table 1. Pattern of antibiotic susceptibility of Salmonella isolates.

Antibiotic	Aerobic Incubation			Anaerobic Incubation		
	R%	[95%CI of R%]	S%	R%	[95%CI of R%]	S%
Amoxicillin	60.9	31.8-55.9	39.1	62.9	35.2-59.4	37.1
Ampicillin	62.3	26.5-50.2	37.7	67.1	33.9-58.0	32.9
Azithromycin	21.7	13.1-33.6	78.3	34.3	23.6-46.7	65.7
Cefoxitin	42	16.6-38.3	58.0	45.7	28.7-52.4	54.3
Cephalexin	58	38.5-62.9	42.0	55.7	40.6-64.8	44.3
Ciprofloxacin	89.9	30.4-54.5	10.1	91.4	33.9-58.0	8.6
Colistin	34.8	24.0-47.3	65.2	38.6	27.4-51.0	61.4
Chloramphenicol	40.6	25.3-48.8	59.4	42.9	26.1-49.6	57.1
Ceftriaxone	30.4	8.6-27.2	69.6	37.1	7.4-25.2	62.9
Gentamycin	30.4	4.5-20.4	69.6	25.7	7.4-25.2	74.3
Imipenem	40.6	13.1-33.6	59.4	44.3	21.1-43.8	55.7
Nalidixic	81.2	51.2-74.7	18.8	71.7	36.6-60.7	28.6
Novobiocin	87	66.4-86.9	13.0	85.7	66.8-87.1	14.3
Streptomycin	37.7	9.7-28.8	62.3	41.4	22.4-45.2	58.6
Tetracycline	81.2	52.7-76.0	18.8	61.4	46.2-70.0	38.6
Sulfa-TMP	59.4	39.9-64.2	40.6	61.4	37.9-62.1	38.6

A comparison of resistance and susceptibility patterns of selected six antibiotic drugs with the greatest difference in the diameter of the zone of inhibition in aerobic and anaerobic incubation conditions is shown in **Figure** 2.

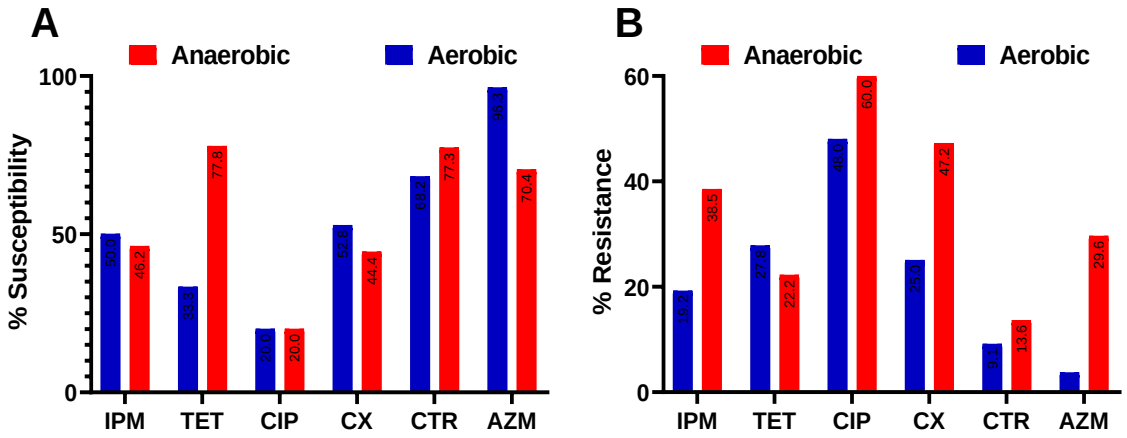

Figure 2. Comparison of susceptibility (A) and resistance (B) of six selected antibiotics (selection was based on the top six largest differences in the diameter of zone of inhibition) in aerobic and anaerobic incubation. The antibiotic molecules in the x-axis are abbreviated as IPM: Imipenem, TET: Tetracycline, CIP: Ciprofloxacin, CX: Cefixime, CTR: Ceftriaxone, and AZM: Azithryomycin.

The highest percentage of resistance was observed against nalidixic acid (63.8%/48.6%) and tetracycline (65.2%/58.6%), trimethoprim/sulphamethoxazole (52.2%/50%), cephalexin (50.7%/52.9%), ciprofloxacin (45.7%/45.7%), amoxicillin (43.5%/47.1%), ampicillin (37.7%/45.7%) and chloramphenicol (36.2%/37.1%) in aerobic/anaerobic condition. Resistance towards gentamicin (10.1%/14.3%) and ceftriaxone (10.1%/14.3%) was the lowest for all isolates. Among sixteen different antibiotics, six antibiotics showed the greatest difference in the diameter of the zone of inhibition in aerobic and anaerobic conditions namely; imipenem, tetracycline, ciprofloxacin, ceftriaxone, azithromycin, and cefoxitin. These six antibiotics were selected for two-way ANOVA analysis in GraphPad Prism vs. 8.0 on a Windows 8.0 platform. The susceptibility analysis showed that the variations contributed by the antibiotic itself were 77.5% (P=0.0976) whereas the variation contributed by the incubation condition was only 0.3191% (P=0.7991). Both of these were statistically non-significant which implied that the susceptibility of the Salmonella isolates to these selected six antibiotics either aerobic or anaerobic incubation did not differ to a greater extent (**Figure** 2A). However, when the resistance of these same antibiotics to the same Salmonella isolates was compared in similar incubation conditions, the results showed that the difference was statistically significant. Resistance analysis showed that the variation contributed by the type of antibiotic on the degree of resistance was 72.73% (P=0.0303) whereas the variation contributed by the incubation condition was 16.09% (P=0.0437). This showed that though the contribution of the incubation on the degree of resistance was low, it was significant as compared to the contribution on the susceptibility (**Figure** 2B).

DISCUSSION

This study demonstrates the antibiotic susceptibility of *Salmonella* isolates and its change in aerobic and anaerobic conditions. *Salmonella* isolated from poultry showed greater resistance to nalidixic (89.5%), tetracycline (80%), ciprofloxacin (64.9%), sulphamethoxazole (42%), trimethoprim (29.8%), and ampicillin (26.3%) (Andoh et al 2016). Resistance to the first-line drugs (chloramphenicol, amoxicillin and trimethoprim-sulphamethoxazole), tetracycline, and nalidixic acid is similar to the results of the studies carried out in Eastern Nepal (Bantawa et al 2019) and other countries like Portugal, Ethiopia,

United States, Belgium, and China (Antunes et al 2003, Asfaw Ali et al 2020, Velasquez et al 2018, Vinueza-Burgos et al 2019, Wang et al 2019). The findings were not unexpected given the indiscriminate and extensive use of antibiotics in the poultry industry as access to antibiotics is easy and can be purchased without a prescription in Nepal. In Egypt (El-Sharkawy et al 2017) and Zimbabwe (Makaya et al 2012) studies show the susceptibility of *Salmonella* to trimethoprim/sulphamethoxazole. The susceptibility could be due to the absence of integron that consists of *dfr*A12 trimethoprim resistance cassette in the *Salmonella* isolated in the study. The antibiotic resistance pattern varies by place or country depending upon the selection pressure contributed by the antibiotic usage in animals, as well as geographical variation in the epidemiology of Salmonella and the regional prevalence of a certain serovar (McDermott et al 2018).

Salmonella isolates in our study were susceptible to azithromycin and ceftriaxone. Minimally used antibiotics retain good susceptibility (Velasquez et al 2018). The extended-spectrum cephalosporins such as ceftriaxone and azithromycin are effective alternatives for the treatment of salmonellosis (Crump et al 2015). Azithromycin has greater penetration to most tissues and attains intracellular concentrations inside macrophages and neutrophils so is considered equivalent or superior to chloramphenicol, and fluoroquinolones (Crump et al 2015). Though the isolates in our study did not show high resistivity against ceftriaxone, the occurrence of resistance has been reported especially in non-typhoidal salmonella strains (Asfaw Ali et al 2020, Crump et al 2015).

Six out of the sixteen antibiotics used in the study, with a greater difference in the diameter of the zone of inhibition, were further analyzed. The analysis showed that the susceptibility pattern of *Salmonella* spp. was not significantly different in anaerobic compared with aerobic conditions. However, the resistance pattern of the same *Salmonell*a isolates for the same drugs was statistically significant. Our study partially agrees with a study by DeMars et al 2016 which reports significant differences in the susceptibility of *Salmonella* and *E. coli* spp. to antimicrobial drugs in anaerobic compared to aerobic conditions. Non-significant variation in susceptibility to the drugs in our study could be due to the smaller sample size. The drugs namely imipenem, cefoxitin, ciprofloxacin, ceftriaxone, and azithromycin showed increased resistance in anaerobic conditions on the other hand tetracycline showed decreased resistance in anaerobic conditions.

Salmonella needs to colonize the distal small intestine or colon to initiate enteric disease. Besides competing with the inhabitant microbes within the intestinal tract, the pathogenic organism is also introduced to a variety of physicochemical signals such as a change in temperature, presence of host antimicrobial peptides, bile salt, pH alteration, and anaerobiosis (Sengupta et al 2014). Aerobic to anaerobic transition is responsible for changes in the central metabolism of the organism which leads to upregulation and suppression of several proteins to adapt to anaerobic metabolism (Encheva et al 2009). Furthermore, *in vivo*, the organism is subjected to nutrient-starved low-oxygen conditions, where its metabolic state resembles that of stationary-phase bacteria grown in the laboratory (Judy et al 2009). The observed differences in resistance percentage of *Salmonella* isolate in aerobic and anaerobic conditions are likely associated with physiological changes such as alteration in bacterial population growth rate, respiration, and metabolism, along with changes in uptake or penetration of antimicrobials within the bacterial cells (DeMars et al 2016) which alters bacterial susceptibility to antimicrobial drugs that inhibit the synthesis of the cell wall of vegetative, growing-to-divide bacteria, such as β lactams and antimicrobial drugs that target major metabolic cell functions, such as macrolides, sulfonamides, and tetracyclines are altered due to modification in metabolic function and population growth rates (DeMars et al 2016).

The anaerobic condition can lead to substantial decreases in the expression of periplasmic transport proteins which are involved in the uptake of amino acids and peptides thereby reducing the uptake of antimicrobials such as azithromycin (Encheva et al 2009). Change in respiration patterns in the anaerobic environment has a crucial role in the uptake and lethality of aminoglycoside (Kohanski et al 2010). Following the initial step of adsorption of drug molecules through electrostatic interaction, changes in membrane potential allow the aminoglycosides to access the cell. Respiration-dependent uptake relies on the activity of membrane-associated uptake of aminoglycosides which is severely limited within bacterial cells under anaerobic conditions (DeMars et al 2016, Kohanski et al 2010). The study of Multidrug Efflux Pump (MdtEF) in *E. coli* identified a dramatic up-regulation of an additional efflux pump, more than 20-folds under anaerobic conditions, regulated by the global transcription factor arcA, resulting in increased efflux activity and enhanced drug tolerance for tetracycline (Blanco et al 2016, Zhang et al 2018).

Salmonella serotypes are resilient microorganisms with a complex genomic system that enables the organism to react and adapt to different harsh environmental conditions at the farm, during processing, and in the gastrointestinal tract of the host (Antunes et al 2016, Crump et al 2015). The bacteria are exposed to different stress factors beyond their normal growth range, one being fluctuation in oxygen gradient through the farm-to-fork passage (Sengupta et al 2014). The transition from an aerobic environment to an anaerobic environment leads to a series of modifications in the physiology and growth rate of microorganisms which indeed alter the effect of antimicrobials in a different culture or incubation environment (DeMars et al 2016, Sengupta et al 2014). The case of disparity between *in vitro* susceptibility and the clinical outcome of the treatments for salmonellosis hinders effective treatments. Hence, the pharmacodynamics studies of facultative anaerobic enteric organisms like *Salmonella* should be carried out in conditions that correctly mimic their natural habitat in the host species (for example, the anaerobic condition). Such studies aid in determining the exact efficacy of antimicrobial drugs in situ conditions, avoiding extensive indiscriminate usage and treatment failure.

CONCLUSION

The findings of our study demonstrate that the susceptibility/resistance of NTS varies in aerobic and anaerobic incubation so it is suggested to undertake the AST in both conditions for the best possible outcomes. Antibiotic susceptibility *in vivo* depends upon the role of oxygen gradients and temporal exposure of enteric pathogens in the host. The misinterpretation of the results obtained from the AST could result in the underestimation or overestimation of antibiotic efficacy in clinical practice. Future research could be directed towards re-evaluating by taking all test conditions (incubation time and inoculation level) into account in providing clinical guidelines and recommendations for chemotherapy and accurately predicting pharmacokinetics and pharmacodynamics in vivo Hence, pharmacodynamics models aiming to evaluate the impact of antibiotics use in enteric bacteria such as *Salmonella* spp. of the treated host should utilize measurements of bacterial susceptibility that are obtained anaerobically to achieve effective antibiotic treatment and control NTS infection.

ACKNOWLEDGEMENTS

The authors acknowledge the support and assistance provided by the technical and administrative staff of the National Animal Health Research Centre during the study period. The funds used in this study were provided by the NARC Project No 542 (2020) entitled “Monitoring of Antimicrobial Resistance in Pathogens Isolated from Food Animals (MARPFA).

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