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Original Article

Drug resistance and susceptibility testing of Gram negative bacterial isolates from healthy cattle with different β – Lactam resistance Phenotypes from Shandong province China

Teste de resistência a drogas e suscetibilidade de isolados bacterianos Gram negativos de bovinos saudáveis com diferentes fenótipos de resistência a β - lactâmicos da província de Shandong, China

S. Arbab[a,b,c], H. Ullah[d], X. Wei[a,b,c], W. Wang[a,b,c], S. U. Ahmad[a,b,c] and J. Zhang[a,b,c]*

[a]Ministry of Agriculture, Key Laboratory of Veterinary Pharmaceutical Development, Lanzhou, China
[b]Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences, Lanzhou, China
[c]Key Laboratory of New Animal Drug Project of Gansu Province, Lanzhou, China
[d]Sichuan University, West China School of Nursing, West China Hospital, Department of Nursing, Chengdu, China

Abstract

The objective of this study was to evaluate the effectiveness of common antibiotics against different microorganisms in apparently healthy cattle in Shandong province and its suburb. A total of 220 nasal swab samples were collected and cultured for bacteriological evaluation. All the bacteria isolates after preliminary identification were subjected to antibiogram studies following disc diffusion method. It was found in the study that *E. coli* is the most commonly associated isolate (21%), followed by *Klebsiella* spp. (18%), *Pseudomonas aeruginosa* (13%), *Salmonella* spp. (15%), *Shigella* spp (12%), and *Proteus* spp (11%). While the antibiogram studies reveled that highest number of bacterial isolates showed resistance to Ampicillin (95%), followed by Augmentin (91%), Cefuroxime (85%) and Tetracycline (95%) of (*Escherichia coli* and *Klebsiella* spp). In the case of *pseudomonas* spp. and *Salmonella* the highest resistance was showed by Ampicillin (90%) followed by Amoxicillin + Clavulanic Acid (80%), Cefixime (90%), and Erythromycin (80%). In *Shigella* spp and *Salmonella* spp highest resistance was showed by Amoxicillin, Ceftazidime, Augmentin (60%), and Amoxicillin + Clavulanic Acid (50%). It is concluded that in vitro antibiogram studies of bacterial isolates revealed higher resistance for Ampicillin, Augmentin, Cefuroxime, Cefixime, Tetracycline, Erythromycin, and Amoxicillin + Clavulanic Acid. The high multiple Antibiotics resistance indexes (MARI) observed in all the isolates in this study ranging from 0.6 to 0.9. MARI value of >0.2 is suggests multiple antibiotic resistant bacteria and indicate presence of highly resistant bacteria.

Keywords: multidrug resistance, healthy animals, infectious agents, antibiotic.

Resumo

O objetivo deste estudo foi avaliar a eficácia dos antibióticos comuns contra diferentes microrganismos em bovinos aparentemente saudáveis na província de Shandong e seus subúrbios. Um total de 220 amostras de esfregaço nasal foi coletado e cultivado para avaliação bacteriológica. Todos os isolados de bactérias após identificação preliminar foram submetidos a estudos de antibiograma seguindo o método de difusão em disco. Verificou-se no estudo que *E. coli* é o isolado mais comumente associado (21%), seguido por *Klebsiella* spp. (18%), *Pseudomonas aeruginosa* (13%), *Salmonella* spp. (15%), *Shigella* spp (12%) e *Proteus* spp (11%). Enquanto os estudos de antibiograma revelaram que o maior número de isolados bacterianos apresentou resistência à Ampicilina (95%), seguido por Augmentin (91%), Cefuroxima (85%) e Tetraciclina (95%) de (*Escherichia coli* e *Klebsiella* spp). No caso de *Pseudomonas* spp. e *Salmonella*, a maior resistência foi apresentada pela Ampicilina (90%) seguida pela Amoxicilina + Ácido Clavulânico (80%), Cefixima (90%) e Eritromicina (80%). Em *Shigella* spp e *Salmonella* spp, a maior resistência foi demonstrada por Amoxicilina, Ceftazidima, Augmentina (60%) e Amoxicilina + Ácido Clavulânico (50%). Conclui-se que estudos de antibiograma in vitro de isolados bacterianos revelaram maior resistência para Ampicilina, Augmentina, Cefuroxima, Cefixima, Tetraciclina, Eritromicina e Amoxicilina + Ácido Clavulânico. Os altos índices de resistência a antibióticos múltiplos (MARI) observados em todos os isolados neste estudo variaram de 0,6 a 0,9. O valor MARI de > 0,2 sugere várias bactérias resistentes a antibióticos e indica a presença de bactérias altamente resistentes.

Palavras-chave: resistência a múltiplas drogas, animais saudáveis, agentes infecciosos, antibióticos.

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*e-mail: infzjy@sina.com; drsafiakandhro@gmail.com
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1. Introduction

Production of extended-spectrum β-lactamases is the most common mechanism of resistance to third-generation cephalosporin's among Enterobacteriaceae family including *Klebsiella pneumoniae* and *Escherichia coli* (Pitout and Laupland, 2008). The recent incidence associated to these bacterial isolates, in companion animals and in livestock, has got the concentration for its monitoring studies in livestock (Smet et al., 2010). β-lactamases producing *E. coli* isolates are now being frequently detected in food-producing animals, which can become infection sources or reservoirs of these pathogenic bacteria and enhance the spread of these bacteria (Carattoli, 2008).

Antimicrobial agents are used therapeutically in animals and humans for the control of bacterial infections and is also being incorporated into commercial livestock feed at sub therapeutic doses for growth promotion (Berghiche et al., 2018). The uses of antibiotics have significantly reduced mortality associated with infectious diseases in 20th century; but unfortunately, their massive and irregulated uses, in animal farming, has led to the emergence of bacteria multidrug resistance (MDR) (Berghiche, 2019). However, the emergence of resistance has been a global challenge in the application of antibiotics in both humans and animals. Regardless of the source, antibiotics resistance has been on raise and recently, has been projected to be among the major killer that will contribute to death of more than 10 million people annually by 2050 if the threat is not contained (de Kraker et al., 2016).

Large amount of antibiotics are used for non-human purposes, which largely exceed use for man and the applications in animal husbandry is not for therapeutic purposes, rather as growth promoters, feed additives and for prophylaxis (Abat et al., 2017; Akbar et al., 2014). Furthermore, it is estimated that the global consumption of antibiotics is approximated to be around 70 to 80% and the projected increase of 67% by year 2030 (Van Boeckel et al., 2015). This could be explained for the quest of large livestock products for profit making in many countries. Although, the use of antibiotics in farming and agriculture is banned in most European countries for prophylaxis, however, the practice of applications of antibiotics in animal husbandry is still common in many countries across the world (Robinson et al., 2016; Woolhouse et al., 2015). The use of antibiotics in animal husbandry results residual of antibiotics in food of animal origins (Galadima et al., 2018). In China, due to the recent boom in agriculture especially livestock breeding, many farmers resort the uses of antibiotics indiscriminately for prophylaxis and as a growth promoter. Furthermore, poor antibiotic stewardship complicated the scenario, hence, this study, aimed to isolate bacteria of public health importance and their resistance profiles in apparently healthy animals.

2. Material Methods

2.1. Ethics statement

All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, and all efforts were made to minimize suffering. This work was supported by grants from the National Natural Science Foundation of China (No: 31872520); China Agriculture Research System (CARS-37).

2.2. Sample collection

The samples were collected from apparently healthy animals which showed no symptom of any illness. A total 220 nasal swab samples were collected with the use of sterile swab stick. The swab was then transferred to its care, labeled and taken to the laboratory.

2.3. Bacterial isolation and identification

Swabs sample were cultured overnight into nutrient broth at 37 ˚Ċ for the determination of microbial growth and then sub-cultured on Blood agar, Chocolate agar, Sorbitol Macconkey Agar, Eosin Methylene Blue Agar, and *Salmonella Shigella* agar plates incubated at 37˚Ċ for 24 hr. Suspected colonies were selected for further analysis of pure culture of gram negative bacteria using standard microbiological techniques of colony identification with the help of Gram staining and biochemical tests following Bergey's manual of determinative bacteriology (Holt et al., 1994).

2.4. Antibiotic susceptibility test

Antibiotic susceptibility testing was done for the identified isolates according to Modified Kirby-Baur disc diffusion method using Muller-Hilton agar and the results were interpreted according to Clinical Laboratory Standards Institute (CLSI, 2017) guidelines. Antibiotic discs were placed over the surface of pre inoculated media using dispenser and gently tap each antibiotic disc onto the surface of the agar with a sterile stick, and incubated at 37°C for 24 hours. The double-disk synergy test with cefotaxime and amoxicillin- Clavulanic Acid disks was used for the screening of extended-spectrum of ß-lactamases.

2.5. Antibiotics used in the study

The Kirby-Bauer disk diffusion susceptibility test was used to determine the sensitivity or resistance of all confirmed isolates to eighteen different antibiotics namely: Ampicillin (AM) (10 μg), Amoxicillin (AMX) (25 μg), Amoxicillin + Clavulanic Acid (AMC) (20/10 µg), Augmentin (AUG) (30 μg), Cefotaxime (CTX) (30 μg), Ceftazidime (CAZ) (30 μg), Cefuroxime (CXM) (30 μg), Ciprofloxacin (CPX) (10 μg), Cefixime (CXM) (5 μg), Cefpodoxime (CP) (10 μg), Ofloxacin (5 μg), Imipenem (10 μg), Tetracycline (30 μ), Gentamicin (GEN) (10 μg), Streptomycin (STR) (10 μg), Erythromycin (ERY) (10 μg), Chloramphenicol (CHL) (25 μg), Nitrofurantoin (NIT), and Cotrimoxazole (STX) (25 μg).

2.6. Determination of Multiple Antibiotic Resistance Index (MARI)

MARI was calculated using the formula, MARI=a/b where "a" is the number of antibiotics resisted and "b" is the total number of antibiotics used in the study. Isolate with MARI value of >0.2 suggests multiple antibiotic resistant bacteria and indicate presence of highly resistant bacteria (Krumperman, 1983).

2.7. Statistical analysis

The graphic representation was performed using Microsoft Office Excel, 2007.

3. Results

3.1. Confirmation of bacterial isolates

Identification of bacterial Isolates was based on the detailed morphological and biochemical profiling, whereas the Gram staining were done for the confirmation of the Gram-negative bacteria. A of total (220) samples were collected from Shandong province, the positive yield growth 206 (93%), of Gram negative bacteria. Whereas *E. coli* was the most frequently recovered (21%), bacteria followed by *Klebsiella* spp. (18%), *Pseudomonas aeruginosa* (13%), *Salmonella* spp. (15%), *Shigella* spp. (12%), and *Proteus* spp. (11%) as shown in (Figure 1), and Supplementary Materials (Table 1).

3.2. Antibiotic susceptibility patterns of the isolates

The antibiotic susceptibility results were recorded as resistant, intermediate, and sensitive following the guidelines of Clinical Laboratory Standard Institute (CLSI, 2011). The resistance revealed that the isolates (*E. coli* and *Klebsiella* spp.) are highly resistance to Ampicillin, Tetracycline, Augmentin, and Cefuroxime, as shown in (Figure 2), and Supplementary Materials (Table 2).

Pseudomonas aeruginosa and *Salmonella* spp. isolates that were subjected to antimicrobial testing and interpreted as resistant, to Ampicillin, Tetracycline, Cefixime, Erythromycin, and Amoxicillin + Clavulanic Acid as shown in (Figure 3), and supplementary (Table 3).

Shigella spp. and *Proteus* spp. isolates showed resistance to at least eight antibiotics. All isolates that were resistant to more than two classes were identified as MDR, Amoxicillin + Clavulanic Acid, Ceftazidime, and Erythromycin as shown in (Figure 4), and Supplementary Materials (Table 4). All the 206 Gram negative microorganisms isolate showed resistance to at least seven antibiotics.

3.3. Determination of Multiple Antibiotic Resistance Index (MARI)

Multiple Antibiotic Resistance index of isolates that exhibited resistance to three or more antibiotics were generated dividing number of antibiotics resistant the total number of antibiotics tested (Table 5).

4. Discussion

Antibiotic resistance has continued to constitute serious problems not only in human but also in animal husbandry, livestock management, and Veterinary (Lawson, 2008). Thus, it is important to assess the resistance profile of

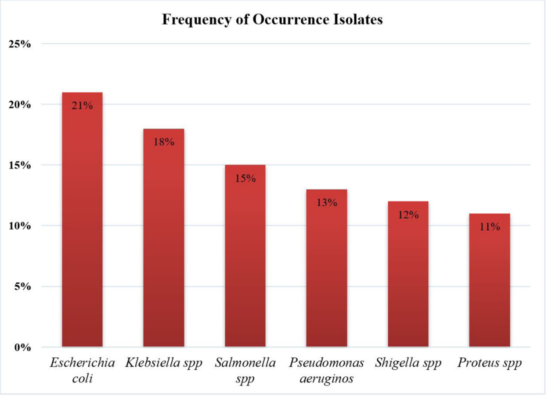

Figure 1. Frequency of occurrence of isolates from different microorganism.

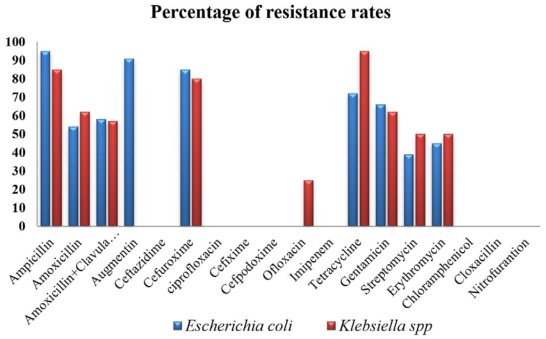

Figure 2. Frequencies of antibiotics resistance in *E. coli* and *Klebsiella* spp.

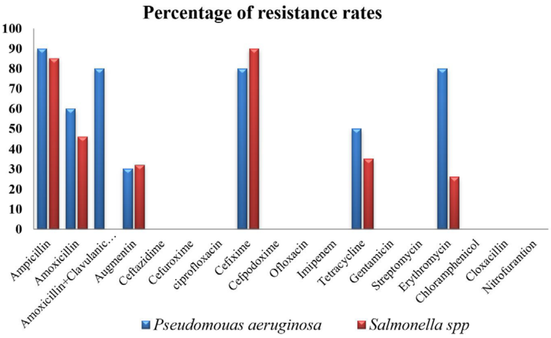

Figure 3. Frequencies of antibiotics resistance in *Pseudomonas aeruginosa* and *salmonella spp*.

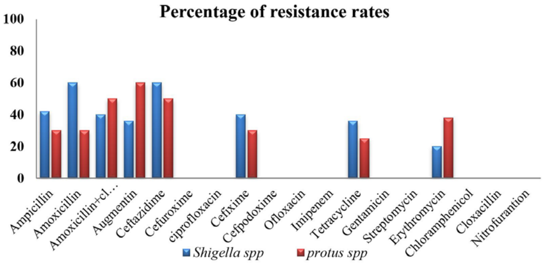

Figure 4. Frequencies of antibiotics resistance in *shigella spp* and *protus* spp.

bacteria among apparently healthy animals as they are common sources of food. We have collected nasal swab samples of cattle to assist the drug resistance profile of isolates from healthy livestock.

The prevalence of bacterial isolates was found 23%, accounting for large proportion. *E. coli* and *Klebsiella* spp. was the most common species isolated in this study, followed by *Pseudomonas* spp (14.5%), and *Salmonella* spp (16.9%), *Shigella* spp. (13.59%) and *Proteus* spp (12.1%) respectively. In recent time, it was demonstrated that *E. coli* was predominant species in healthy animals (Oloso et al., 2018), these finding are in agreement with the current study. In a similar manner, high rate of *E. coli* was reported in cows from Jordan (Zhang et al., 2018). These finding are also consistent with other reports where *E. coli* was observed as the predominant species in healthy animals (Oloso et al., 2018).

Overall, the high prevalence of *E. coli* and occurrence of other bacterial isolates can be explain by being the members of normal flora in animals, however, occurrence of *E. coli*, *Salmonella* spp. *Pseudomonas* spp. *Proteus* spp. and *Klebsiella* spp. is a pointer to high burden that have potential risk to animals and human health.

In this study, disk diffusion methods were employed for antibiotic sensitivity testing. The major finding in the present study is the presence of multiple drug resistant *E. coli* in animals to commonly in use antibiotics such as, Ampicillin (95%), Augmentin (91%), Cefotaxime (85%), Tetracycline (72%), Gentamicin (66%), and Amoxicillin + Clavulanic Acid (58%). This observation reiterates the finding in other studies that have reported antibiotic resistance among bacteria especially *E. coli* isolated from cattle and other animals is increasing at an alarming rate (Ogunleye et al., 2008).

In the present study, MDR *Klebsiella* spp. was found to be 95% followed by Tetracycline, Ampicillin (85%), Cefuroxime (80%), and amoxicillin+ Clavulanic Acid and Gentamicin (62%). In a study from China, high rate resistant *Klebsiella* spp. was reported to be 93.4% (Cheng et al., 2018). Similar studies documented MDR *Klebsiella* spp. in cattle (Osman et al., 2014; Tasnim and Islam, 2015). Similar results were reported with high resistance *Escherichia coli* (35%), *Shigella* (33%) and *Salmonella* spp. (25%) (Arbab et al., 2021). MDR isolates in healthy food animals is a serious threat to food safety and public health, as there is well established evidence of resistance transfer through food animals to humans (Chiou et al., 2017).

Isolates originating from this study were shown to be multidrug resistant. The trends in resistance pattern showed that *Salmonella* spp. Cefixime and Ampicillin (85-90%) was more resistance than other isolates. This finding shows similar pattern of high resistant *Salmonella* spp in previous study in China, where the MDR *Salmonella* spp. reported to be 80% in food animals when tested against 17 common antibiotics (Lu et al., 2014). Previously, report from Ghana also reported high prevalence of *Salmonella* spp. (66.7%) where MDR of the isolates were reported to be 52.8% (Osei Sekyere and Adu, 2015). Across the world, prevalence of *Salmonella spp.* were reported in varying degree with very low prevalence in Europe (2%) than other continents which is in contrast with the current study (Gutema et al., 2019). This variation of occurrence could be accounted for the methods the cattle are handled in different geographical regions.

Resistance pattern showed that *Pseudomonas aeruginosa* to Ampicillin (90%), Amoxicillin + Clavulanic Acid (80%), and Cefixime and Erythromycin (80%), were intermediate resistant than other isolates. MDR *Pseudomonas aeruginosa* has been reported with varying degree of resistance to different antibiotics, (93.8%) to beta-lactam and least resistance to Fluoroquinolone (16%) (Beier et al., 2015). *P. aeruginosa* as adaptive pathogen, exhibiting multidrug pattern is worrisome. We reported 70% *P. aeruginosa* resistance which is in agreement with previous studies depending on the class of antibiotics (Elshafiee et al., 2019). Similar multidrug resistance *Pseudomonas aeruginosa* has been previously reported in healthy cattle from France and elsewhere (Cheng et al., 2018).

In this study, two other MDR isolates were also reported, *Shigella spp* and *Salmonella* spp showed low resistant compared to other isolate Amoxicillin, Ceftazidime and Augmentin (60%). Researchers reported high levels of resistance for tetracycline. No resistance was observed to Gentamicin, Amikacin, Nalidixic acid, Ofloxacin, Pefloxacin, Cefotaxime, Cefoxitin and Imipinem (Mokhtari et al., 2012). Amongst the food strains, one strain was MDR with resistance to five different antibiotics. There was also a high sensitivity of *Shigella spp*. strains isolated from the control group to antimicrobial agents (75%, three of four) (Mokhtari et al., 2012).

The multiple antibiotic resistance index (MARI) of the isolates recovered in the present study indicate multidrug resistance in nature. The MARI value > 0.2 suggesting multidrug resistance, due to high risk application and contamination of antibiotics (Joseph et al., 2017). These findings demonstrate that the cattle were exposed to multiple antibiotics. On the other hand, a lower MARI index ranging 0.3-0.6 was reported in South African study in food animal (Iwu et al., 2016). Comparable finding also reported lower MARI (0.31) in healthy livestock from South Africa (Nsofor and Iroegbu, 2013). This can be explained by the sample size and antibiotic regulation in the study area, among other factors.

Lack of epidemiological variables, such as history of antibiotic use, to assess the risk factors of exposure and development of resistance and sample size are among the limitation of this study. In addition, molecular analysis could not be performed to determine the resistant gene due to funding constraint. Thus, future studies are required to explore molecular nature of the multidrug resistant genes and the risk factors associated with harboring of drug resistant bacteria in healthy animals in the study area.

5. Conclusion

It is concluded that, *Escherichia coli* and *Klebsiella* spp. were the most prevalent bacteria in healthy cattle followed by *Salmonella* spp, *Pseudomonas aeruginos*, *Shigella* spp and *Proteus* spp. The bacterial species, which were isolated, are resistant to single of multiple antimicrobial used. The diversity of microorganisms observed in this study support the need of microbial culturing from nasal swab and its antibiotic susceptibility determination for adequate chemotherapy. We also suggest that the routine investigate and monitoring of cattle for bacterial culture and sensitivity studies in various parts of China.

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Supplementary Materials

Supplementary material accompanies this paper.

Table 1. Distribution of gram-negative organisms isolated from cattle.

Table 2. Frequencies of antibiotics resistance of Escherichia coli and Klebsiella spp isolates.

Table 3. Frequencies of antibiotics resistance of Pseudomonas spp and Salmonella spp isolates.

Table 4. Frequencies of antibiotics resistance of Shigella spp and Protus spp isolates.

Table 5. Multiple drug resistance indexes (MARI) of the isolates.

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