Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 9 (1): 51-58, 2014
ISSN 1816-4935 / DOI: 10.3923/jm.2014.51.58

Multi Drug Resistance of *Pasteurella* spp. Isolated from Sheep and Goats in Iran

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ABSTRACT

One of the most frequent causes of respiratory infection and death in sheep and goats is *Pasteurella multocida*. Among the antibiotics, penicillin is the important member of the beta-lactam group has a wide spectrum of activity against *Pasteurella* strain. Although, *Pasteurella* usually susceptible to penicillin, tetracycline or chloramphenicol, during the several years ago, drug-resistant to *Pasteurella* strains have been encountered. The aim was evaluation of resistance pattern of *Pasteurella* to antimicrobial agents. A collection of 64 *P. multocida* strains were evaluated against 23 antimicrobial agents using two methodology, E test and disk diffusion. Nearly fifteen percent of *P. multocida* was significantly resistance to penicillin. Multi drug resistance was also observed among the isolates. E test MICs results were always more susceptible than those obtained by disk diffusion at the end of the MIC ranges. Penicillin resistance of *P. multocida* isolated from sheep and goats and also resistant to clindamycin and oxacillin have been reported. Eventually, use of Penicillin could potentially give rise to antibiotic resistance in *P. multocida* and should be considered as a risk factor for treatment of human pasteurellosis.

Key words: *Pasteurella*, antibiotic resistant, sheep, goats, Iran

INTRODUCTION

Antimicrobial drugs have been widely used in human and veterinary medicine for more than 50 years with benefits to both human and animal health. Antimicrobial medicines are used against bacteria in human and animal food product during the 20th century (FDA, 2010). The introduction of antibiotics was one of the most important developments in modern medicine (Gottlieb and Nimmo, 2011). Following the use of antibiotic treatment in food animal such as cattle, sheep and goats developed with more than half (52%) of those bacteria resistant to antibiotics (Van Looveren *et al*., 2001). International and US public health agencies have targeted antibiotic resistance as an emerging public health concern and one of the greatest threats to human health worldwide (Barza and Travers, 2002; Spellberg *et al*., 2008).

Resistance which enables microbes to escape being killed by antimicrobial (including antibacterial, antiviral, antifungal, etc.) drugs, undermines physicians' ability to treat serious and life threatening infections. Antibiotic resistant usually microorganisms are able alive after exposure to antibiotics, therefore physicians are not able to treatment dangerous infection illness (Spellberg *et al*., 2008; Boucher *et al*., 2009).

Veterinary Investigation Disease Analysis (VIDA) statistics have revealed that, over the past ten years or so, one of the most commonly diagnosed infectious causes of disease or death in sheep, in descending order, have been pneumonia due to the *Mannheimia* spp./*Pasteurella* spp. Veterinary Investigation Disease Analysis (VIDA) have indicated during ten years ago *Mannheimia* spp./*Pasteurella* spp. are the most representation causes of pneumonia in sheep and goats (Brogden *et al*., 2001). Many animals including sheep and goats are able to become infected from this particular disease leading to pneumonia (Kilonzo-Nthenge *et al*., 2008). Young lambs are the most susceptible to infection from. The most susceptible animal to infection is young lamb because of weak immune system (Bruere *et al*., 2002).

Antimicrobials such as Penicillin are still the drug of choice to control *Pasteurella* (P) *multocida* and *P. hemolytica* infection in animals (Kehrenberg *et al*., 2001a; Schwarz *et al*., 2004). It is the important members of beta-lactam group of antibiotics licensed to treat diseases related to *Pasteurella* sp. in the sheep and goats in Iran (Tehrani *et al*., 2004).

Low doses of antibiotics in animal feed over a long period of time contribute to the growth of antibiotic resistant bacteria that can be transferred to humans and is a greater risk to public health. Exposing bacteria at low doses of antibiotic for long periods growth promoting antibiotic resistant and is a human health risk (Prescott *et al*., 2000; Ismail, 2004). Generally, *P. multocida* isolates are susceptible to most of the widely used commercial antimicrobial agents but their excessive and unjustified use accelerates the emergence of resistant strains. Anti microbial therapy is effective tool for *P. multocida* infection, but using too much enhanced the risk of resistant stains (Ismail, 2004; Sellyei *et al*., 2009). In the last few years, many animal isolates of Pasteurella spp. were found to be resistant to antibiotics. It has been found over the last few years, resistant to antibiotics were found in many animal isolates of *Pasteurella* spp. (Sellyei *et al*., 2009). Resistance to beta-lactam groups has occasionally been reported (Fales *et al*., 1982; Kehrenberg *et al*., 2001b). Due to increasing antibiotic resistance, particularly major bacterial pathogens, accurate antimicrobial susceptibility testing results are vital for both animal care and public health surveillance (Kiehlbauch *et al*., 2000; Reynolds *et al*., 2003).

There are several established methods of antibiotics susceptibility tests used in different studies and different countries (Reynolds *et al*., 2003). Many of them, including disk diffusion, agar dilution, broth micro dilution and antibiotic gradient disks (Jorgensen, 1993; Donabedian *et al*., 2003). E test is convenient and widely used for susceptibility testing of several antibiotics on a large number of bacterial isolates in a short time. E test is appropriate used for study large number of antibiotics on bacterial isolates at one time (CLSI, 2009). Disk diffusion (Kirby-Bauer) is one of the most commonly used antimicrobial susceptibility testing methods among diagnostic laboratories. Disk diffusion (Kirby-Bauer) is most used to determine antibiotic sensitivity testing in diagnostic laboratories (Jorgensen and Ferraro, 2009; Van Belkum and Dunne, 2013). These methods are a well-established procedure and are accepted by the National Committee for Clinical Laboratory Standards (NCCLS, 2000).

We investigated the antibiotic susceptibility against a very large contemporary collection of *P. multocida* in sheep and goats. We also evaluated the correlation between the susceptibility testing results generated by disk diffusion and Etest (AB Biodisk, Solna, Sweden) for 23 antimicrobials. In the current study the antibiotic resistant were evaluated by disk diffusion method and compared with E test for 23 antimicrobial agents. The aim was also to evaluate

whether and how the new species related breakpoints may influence the detection decrease antibiotic susceptibility among *Pasteurella* strains.

MATERIALS AND METHODS

Sample: A collection of 64 *P. multocida* strains isolated from sheep and goats in different parts of Iran were examined.

PCR assay: This collection has been well characterized by PCR relation to kmt1 (all pass), hydD-hydC (Cap A), dcbF (Cap D) and toxA (Dermo Necrotic Toxin) genes in our previous study (Sahragard *et al*., 2011). All isolates harboured toxA gene and belonged to capsular type A.

Antibiotic test: In addition, all *P. multocida* strains were evaluated by the E test methodology against Penicillin, Streptomycin, Gentamicin, Enrofloxacin, Ceftriaxone, Ampicillin, Flumequine, Lincospectin, Tetracycline, Oxacillin, Carbenicillin, Cloxacillin, Cefotaxime, Furaltadone, Neurofloxacin, Clindamycin, Neomycin, Nalidixic acid, Amoxicillin, Ceflotin, Cephalexin, Erythromycin and Oxytetracycline using two inocula (0.5 and 2 McFarland standards) (CLSI, 2009). E test strips were used in according to manufacturer's practice. Mueller-Hinton agar plates were inoculated with a 0.5 McFarland standard of the *P. multocida* isolates. Four E test strips were applied to the surface of the plate in an equidistance radial manner, with the lowest concentration toward the centre. Plates were incubated under the same condition as for agar dilution. Minimum inhibitory concentrations (MICs) were read directly from the test strip at the point where the zone of inhibition intersected the MIC scale on the strip. The concentration gradient of each antimicrobial agent on the E test strips was 0.016-256.0 mg L^{-1}. Disk diffusion tests with 30 μg of all antibiotics disks were performed on all 64 isolates according to the method published by the CLSI for 100 mm Mueller-Hinton agar plates (CLSI, 2006, 2009). The quality control strains, PMSHI (Accession No.: JF694003.1), were tested along with every set of tests.

Data analysis: MIC agreement between the two methods was defined. The following MIC breakpoints defined by the NCCLS for the Pasteurelaceae were employed.

RESULTS

None of the isolates showed resistance to 20 antibiotics (Table 1). The percentages of isolates resistant to antibiotics were 15.62% to Penicillin, 34.37% to Clindamycin and 28.12% to Oxacillin. A scatter gram illustrating the disk diffusion zone diameter results for antibiotics is shown in Fig. 1. Fifty four strains were considered no susceptible to penicillin when tested by the disk diffusion method using the current CLSI interpretation criteria. However, these isolates had inhibition zone diameters of > = 16 mm and were considered susceptible according to CLSI breakpoints for the disk diffusion test (> = 15 mm). A scatter gram illustrating demonstrated the inhibitory zone diameter varied from 10 to 44 mm among the 64 *P. multocida* strains.

Based on MICs from E test, *Pasteurella* isolates (n = 64) demonstrated the significantly high resistance rate ($p<0.05$) to Clindamycin (34.37%), Oxacillin (28.12%) and Penicillin (15.62%) (Fig. 2). Multidrug resistance was also observed among the *Pasteurella* isolates (Fig. 3).

For each antimicrobial agent, E test MICs were always one in two dilutions lower than those obtained by disk diffusion at the susceptible end of the MIC ranges. On the other hand, the E test tended to yield much higher resistant MICs than those measured by disk diffusion at the resistant

end of the MIC ranges ($p<0.05$). The overall agreement of MICs between the two methods was 79.8%, ranging from 45% with Erythromycin to 92.4% with Penicillin. Oxacillin and Clindamycin MIC agreement between the two methods was 56.3 and 61%, respectively.

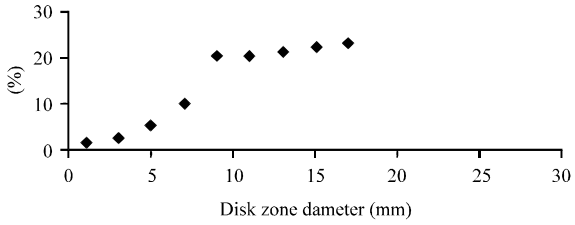

Fig. 1: Thirty two isolates were shown the diameter zone between 10 to 20 mm while 32 of them resistant to Penicillin. Scattergram showing the disk zone diameter results. Thirty two isolates were shown the diameter zone between 10 to 20 mm while 32 of them resistant to Penicillin

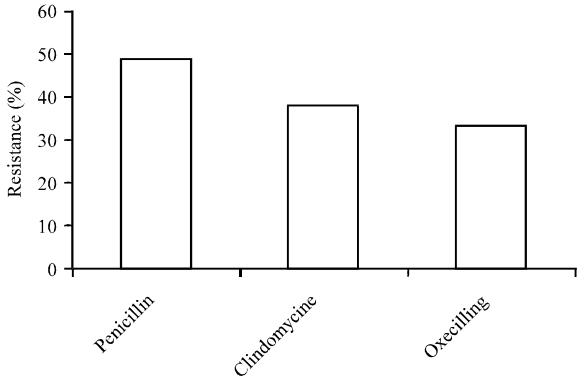

Fig. 2: Antibiotic susceptibility of 64 isolates of *P. multocida*. Penicillin was shown resistant to more than 50% of isolates. Antibiotic susceptibility of 64 isolates of *P. multocida*. Fifty percent of isolates were resistant to Penicillin. But around 30% were resistant to Clindomycin and Oxacillin

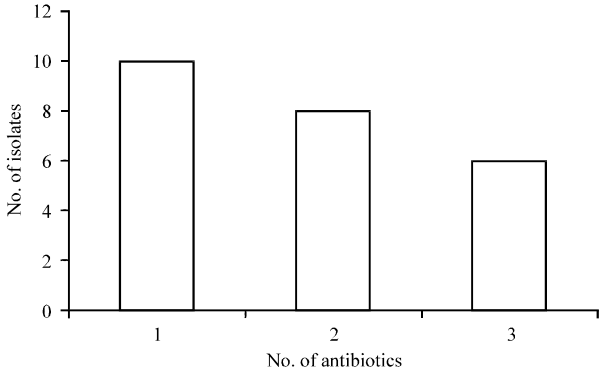

Fig. 3: Multi drug resistance to *P. multocida*, bar 1: Ten isolates resistant to one antibiotic, bar 2: Eight to two, bar 3: Five to three and bar 4: Four to four. Multi drug resistance of *P. multocida* to three antibiotics

Table 1: Antimicrobials and resistance breakpoints used in antimicrobial susceptibility test for *Pasteurella multocida*

	Zone of inhibition (mm)		
Antibiotics	Range	Median	No. of isolates (n = 64) (%)
Gentamycin	10-21	17	24 (37.5)/S[a]
Sterptomycin	6-14	10	50 (78.12)/S
Enrofloxacin	31-51	44	26 (40.62)/S
Ceftriaxon	29-43	37	22 (34.37)/S
Ampicillin	6-14	10	20 (31.25)/S
Flomoquine	23-37	30	18 (28.12)/S
Penicillin	0	0	10 (15.62)/R[b]
Linco spectin	13-28	20	16 (25)/S
Tetracyclin	12-28	20	14 (21.78)/S
Oxacillin	0	0	189 (28.12)/R
Carbenicelen	23-36	30	32 (50)/S
Cloxacillin	7-18	14	36 (56.25)/S
Cefotoxin	11-27	20	8 (12.5)/S
Furaltadon	14-28	20	30 (46.87)/S
Neurofloxacin	26-36	30	2 (3.12)/S
Clindomycin	0	0	22 (34.37)/R
Neomycin	10-20	15	46 (71.87)/S
Nalidoxic acid	15-25	20	4 (6.25)/S
Amoxicillin	11-20	15	24 (37.5)/S
Oxytetracyclin	12-27	15	22 (34.37)/S
Ceflotin	3-16	10	10 (15.62)/S
Cefalexin	8-16	13	14 (21.78)/S
Eryteromycin	10-19	15	26 (40.62)/S

S: Susceptible b, R: Resistant

DISCUSSION

Although, the frequency of resistance varies by area and country, several investigators have been reported a frequency of Penicillin resistance in *Pasteurella* (Okamoto *et al*., 2002; Citron *et al*., 2005). Consequently, it has been recognized that there is a need to test *Pasteurella* and other unusual organisms for antimicrobial resistance. Therefore, there is necessary to evaluate the antimicrobial resistant pattern of *Pasteurella* (Citron *et al*., 2005). Multidrug-resistance (Penicillin, Erythromycin, Oxacillin and Clindamycin) was observed in *P. multocida* that was isolated from sheep and goats in southern Iran. Recent reports have cited evidence for an increase in the incidence of Penicillin resistant throughout the world (Kilonzo-Nthenge *et al*., 2008). The Antibiotic resistance in *Pasteurella* in food animals is leading to increased for treatment of human pasteurellosis and increased public health threat (Graham *et al*., 2007).

However, there were no significant differences noted on disk diffusion method and E test, but the disk diffusion was an easy and inexpensive method for the susceptibility testing. All 64 *P. multocida* strains were highly susceptible to at least one antimicrobial agents with MIC<0.03 mg L^{-1}. The vast majority of the *P. multocida* strains were also susceptible to Enrofloxacin followed by Ceftriaxone, Flumequine, Carbenicelen and Neurofloxacin. A nationwide survey reported in Japanese Journal reported that 40% of *Pasteurella* were resistant to Penicillin G and 53.7% of the same bacteria were resistant to Erythromycin (Okamoto *et al*., 2002).

The correlation between the E test and disk diffusion method, as compared in this study, varied depending on the antimicrobial agent tested. The E test and disk diffusion were the most practical and preferable method for susceptibility testing of *Pasteurella* and the overall exact agreement was 82.9%.

In conclusion, these data indicated that sheep and goats are reservoirs of *P. multocida* those are resistant to antibiotics. Multi drug resistance in food animal pathogens is certainly a public health concern and reinforces the need for more prudent use of antibiotics by farmers, veterinarians and physicians. Therefore, a continued development of methods to reduce the risk of pathogens in food producing animals is critical.

In summary, only the use of a standardized methodology in combination with veterinary specific breakpoints as recommended in the NCCLS document allow a reliable assessment of the number of antimicrobial resistant strains among *P. multocida* strains in Iran. Penicillin was used as an effective choice for *P. multocida* in Iran and this is the first report of Penicillin resistance.

ACKNOWLEDGMENTS

We are indebted to Dr Mohammad H. Hosseini for his antibiogram assistance. This study was supported by the Project Fund (Grant No. 89069-18-14-2).

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