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Isolation, Identification and Antibacterial Resistance Spectrum of Bacterial Pathogens of Respiratory Tract Infection in a Large-Scale Cattle Farm

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ABSTRACT

The current study aimed to report the bacterial pathogens and their antibacterial resistance spectrum causing bovine respiratory tract infections in a large-scale cattle farm. Nasopharyngeal swabs were collected from animals (n=122) having clinical manifestations of respiratory tract diseases. Standard culture procedure followed by the Kirby-Bauer disc diffusion assay was applied to calculate the antimicrobial resistance profile of bacterial isolates. The survey results showed that bronchitis and bronchopneumonia were commonly occurring respiratory diseases in cattle. The highest incidence of bacterial organism *Staphylococcus aureus* (83.6%), followed by *Streptococcus pneumoniae* (80.3%) and *Klebsiella pneumoniae* (77.0%) were recorded in the respiratory tract samples. *Pasteurella multocida, Bacillus obstructivus* and *Mycoplasma alkalescens* exhibited 100% resistance against penicillin, while *Bacillus subtili* and *M. alkalescens* showed 100% resistance against tetracycline. *Mycoplasma dispar, B. subtilis, M. alkalescens, B. obstructivus,* and *Stah. aureus* against cefoxitin; and *M. alkalescens,* and *Micrococcus luteus* against cefoperazone exhibited ≥90% resistance. Overall, the majority of bacterial isolates exhibited ≥70% resistance against many antimicrobials. The antimicrobial spectrum profiles whistle an alarming situation for the regulatory bodies to cut the non-judicial use of antimicrobial agents.

INTRODUCTION

China is contributing about 3.8% of world raw dairy milk production, with an estimated volume of 32 million tons during the year 2019. Dairy cattle are facing many infectious pathogens associated with numerous body systems like respiratory, gastrointestinal, urogenital etc. (Silva and Bittar, 2019). Respiratory tract infections account for 6% of total global infections (Ghimire *et al*., 2022). An upsurge in respiratory diseases has been reported in winter; while other contributing factors include unhygienic bedding, environmental pollution, malnutrition and managemental issues (Kumar *et al*., 2014).

A number of infectious agents were recognized as etiological agents of respiratory diseases, including bacteria, virus, yeast, and protozoans. Bacterial infections are frequent as compared to other infections; however, they affect a smaller group of the population (Carvajal and Perez, 2020). It has been estimated that normal microbiomes of respiratory tract like *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae* etc., could be involved in respiratory tract infections (Prat and Lacoma, 2016). *Mannhemia haemolytica, Pasteurella multocida*, and *Histophilus somni* were isolated as common pathogens of bovine respiratory tract infections (Confer, 2009). Though, these pathogens are the commensals of nostril and nasopharynx (upper respiratory tract) in healthy cattle, and became opportunistic when host defenses are compromised (Timsit *et al*., 2013). *Mycoplasma dispar, Mycoplasma bovis* and *Mycoplasma bovirhinis* were isolated from sick cattle as well (Friis, 1980). Co-infection of many of these bacterial pathogens along with potential viruses may contribute to bovine respiratory disease (BRD), one of the most devastating diseases of the cattle industry globally (Gaudino *et al*., 2022).

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Since the beginning of the 21st century, the problem of antimicrobial resistance is becoming alarming day-by-day. Studies have demonstrated the association between antibiotic use and prevalence of resistance in microbial organisms (Catry *et al*., 2016; Donaldson *et al*., 2006). Data from food-producing animal farms have demonstrated that many farmers use a deviated dose of antimicrobials than those labeled on the leaflet (Timmerman *et al*., 2006), which potentially convert microbiota into bugs (Dewulf *et al*., 2007).

Many variations in antimicrobial resistance profiles of Enterobacteriaceae, Pasteurellaceae and other pathogenic bacteria have been observed between different bovine herds (Tikofsky *et al*., 2003; Catry *et al*., 2005; Bokma *et al*., 2020; Haley *et al*., 2020). It potentially reflects the antimicrobial use in any particular area, and may alarm the various stakeholders of clinical relevance of antibiotic resistance. The purpose of the current investigation was to isolate, identify and explore the antibacterial resistance spectrum of bacterial isolates of bovine respiratory tract pathogens in a large-scale cattle farm.

MATERIALS AND METHODS

Study approval

The study layout was discussed with the farm manager and formal approval was obtained before the start of sampling collection. The samples were collected with the help of on-farm veterinarians and disease diagnosis was also done by a panel discussion of all veterinarians. The institutional approval was obtained from the Animal Ethics Committee of Shihezi University (Approval No. A2022-14403).

Study design and sampling

The study was carried out in the fall of 2022 (November-December) at a large-scale cattle farm that was owned by a large commercial dairy company in China. The farm has Holstein dairy cattle and is located in Xinjiang Uygur Autonomous Region (XUAR) in Northwest China. The farm has fully managed the conditions of intensive dairy farming and all animals were fed silage rather than left to graze. Immature animals (neonatal calves, and one-month-old calves) have separate sections with special care, management and feeding protocols.

Nasopharyngeal swab samples were collected from animals (n=122) suffering from respiratory tract diseases with numerous clinical signs like coughing, sneezing, fever above 40 ºC, difficulty breathing, etc. The animals were restrained properly in standing position and 90% alcohol was applied to nostrils as a disinfectant before sample collection. Sterile transport swabs (Transystem, Copan, Brescia, Italy) were introduced medioventrally in the nasal cavity and after rotating several times were taken out and placed in a transport medium. Within 12h samples were transported to the laboratory and processed for bacteriological investigation.

Bacterial growth

Swabs were inoculated on various general and selective culture media (Oxoid, Ltd., UK) like blood agar, nutrient agar, MacConkey agar, Brilliant Green agar, de-Man-Rogosa Sharpe agar, Eosin Methylene Blue agar and pleuropneumonia-like organism agar, etc. After inoculation, the Petri dishes were incubated aerobically at 37±1ºC for 24-72 hours. For slow-growing organisms like *Mycoplasma* or *Mycobacterium*, the incubation was maintained up to 7 days (Van Driessche *et al*., 2017; Kabir *et al*., 2022). The identification of bacterial isolates was done according to Quinn *et al*. (1992). All analyses were carried out in triplicates and pure cultures were stored for antimicrobial susceptibility testing.

Antibiotic susceptibility testing

The Kirby-Bauer disc diffusion assay was applied to calculate the antibiotic susceptibility patterns of bacterial isolates following the guideline of the Clinical and Laboratory Standard Institute (CLSI, 2021). A single loopful of each pure culture was suspended in Mueller Hinton Broth (Oxoid, Ltd., UK) and cultured on Mueller Hinton agar plates after standardizing to 0.5 McFarland standards. The plates were incubated at 37±1ºC for 24h then zones of inhibition were measured. The breakpoints of CLSI for the tested antimicrobials were used to calculate the susceptibility patterns of bacterial isolates.

Statistical analysis

The data was computed in Excel (Microsoft Inc., USA) Spread Sheets. The prevalence of bacterial organisms in respiratory tract samples and their resistance spectrum were calculated in percentages.

RESULTS AND DISCUSSION

In beef and dairy cattle, respiratory diseases result in massive economic losses that demand deep insight into etiological agents like stressors (e.g., transportation, weaning, dietary changes, etc), the interaction between the host and microbiome, intrinsic immunity as well as chronic sub-clinical inflammation in airways that may lead to the occurrence of respiratory diseases. Next-generation sequencing data have shown that there is a great variation in the upper and lower respiratory tract microbiome that potentially convert into pathogens due to stressors (Chai *et al*., 2022). The genera associated with common respiratory

tract infections like *Mycoplasma*, *Pasteurella* and *Mannheimia* were observed in the nostrils of healthy cattle (Nicola *et al*., 2017), while *Pasteurella, Mycoplasma, Histophilus,* and *Mannheimia* were reported in the nasopharyngeal samples of both BRD-affected and healthy cattle (Zeineldin *et al*., 2017).

The incidence of *Staph. aureus* was highest (83.6%) in the respiratory tract samples, followed by *Strep. pneumoniae* (80.3%), *Klebsiella pneumoniae* (77.0%). Other bacteria that were found in the respiratory tract were *Mycoplasma bovis* (39.344), *Enterococcus faecalis* (31.148), *Klebsiella pneumoniae* (77.049), *Pasteurella multocida* (49.180), *Bacteroides pyogenes* (36.066), *Enterococcus faecium* (45.902), *Clostridium perfringens* (60.656), *Mycoplasma dispar* (26.230), *Bacillus subtilis* (62.295), *Pseudomonas taetrolens* (42.623), *Mycoplasma alkalescens* (29.508), *Bacillus obstructivus* (24.590), *Strep. pneumoniae* (80.328), *Staph. aureus* (83.607), *Micrococcus luteus* (60.656). While *Mannheimia haemolytica* (19.6%), followed by *H. somni* (14.7%) were recorded with the least incidences in respiratory tract infection samples of cattle. *H. somni* and *P. multocida* are common pathogens of bovine respiratory tract infections and their incidence is usually expected less than *M. haemolytica* (Anholt *et al*., 2017; Welsh *et al*., 2004). *Bacillus* and *Acinetobacter* are two bacterial genera that are regarded as microbiota of the nasopharynx and lower respiratory tract of feedlot cattle (Zaheer *et al*., 2013; Zeineldin *et al*., 2017). The high incidence of these genera in the respiratory tract infections could reflect microbial seeding of lungs as a result of regurgitation of feedstuffs during rumination and the formation of aerosols during eructation in ruminants. Bacterial species like *Enterococcus faecium, S. pneumoniae, Enterococcus faecalis*, and *Bacillus* spp., had a relatively high abundance in dairy cattle (Klima *et al*., 2019). *S. pneumoniae* is associated with pneumonia in animals and humans (Borsa *et al*., 2019), whereas, *Enterococcus faecium, Enterococcus faecalis*, and *Bacillus* spp. were regarded as commensal organisms of animal and human gut (Makarov *et al*., 2022).

Studies have shown that respiratory diseases are the 2nd leading cause of death in bovines after gastrointestinal diseases. The prevalence of these diseases was reported 4 to 80% in cattle in recent literature (Pratelli *et al*., 2021; Gaudino *et al*., 2022). The study of Klima *et al*. (2019) dealt with BRD and found that bronchopneumonia was the most prevalent infection among bovine respiratory tract diseases followed by fibrinous bronchopneumonia. Out of 18 observed cases, only one was diagnosed with *Mycoplasma pneumoniae*. During the current investigation, bronchitis followed by bronchopneumonia was recorded as the most common respiratory disease of cattle; while fibrinous bronchopneumonia and *Mycoplasma pneumoniae* were recorded as the least prevalent respiratory diseases of dairy cattle (Table I). *M. haemolytica* was observed as the most prevalent organism in numerous respiratory tract diseases. This finding is in agreement with previous studies that reported that *M. haemolytica* is a common isolate of all types of respiratory tract infections in bovines (Panciera *et al*., 2010; Anholt *et al*., 2017).

Table I. Veterinary diagnosis, number of cases and isolated bacterial species from respiratory tract infections.

Diagnosis	No. of cases	Isolated bacterial species
Chronic bronchopneumonia	14	*Mannheimia haemolytica, Enterococcus faecalis, Klebsiella pneumoniae, Mycoplasma dispar, Bacillus obstructivus, Streptococcus pneumoniae, Staphylococcus aureus*
Bronchopneumonia	22	*M. haemolytica, Mycobacterium bovis, K. pneumoniae, E. faecalis, Pseudomonas taetrolens, B. obstructivus, S. pneumoniae, S. aureus, Micrococcus luteus*
Bronchitis	28	*M. haemolytica, P. multocida, H. somni, E. faecium, C. perfringens, B. subtilis, P. taetrolens, B. obstructivus, S. aureus, M. luteus*
Rhinitis	10	*E. faecalis, E. faecium, B. subtilis, S. aureus, M. luteus*
Fibrinous pneumonia	16	*M. haemolytica, K. pneumoniae, H. somni, M. dispar, P. taetrolens, S. pneumoniae, M. luteus*
Acute suppurative bronchopneumonia	9	*K. pneumoniae, B. pyogenes, C. perfringens, M. alkalescens, S. pneumoniae, S. aureus*
Tracheitis	13	*M. haemolytica, P. multocida, H. somni, E. faecium, B. subtilis, S. aureus, M. luteus*
Fibrinous bronchopneumonia	4	*M. haemolytica, P. multocida, B. pyogenes, E. faecalis, B. obstructivus, S. pneumoniae*
Mycoplasma pneumonia	6	*M. bovis, E. faecium, M. alkalescens, M. luteus*

Table II. Antibacterial resistance percentages of bacterial isolates of respiratory tract infection.

Bacterial isolates (No.)	Antibacterial discs (potency, µg)											
	Gen (10)	Sp (100)	Ch (30)	Cip (5)	Nor (10)	Ery (15)	Lin (2)	Tet (30)	Pen (10)	Cef (30)	Ce (75)	Am (10)
Mannheimia haemolytica (24)	12.5	4.16	29.16	33.33	37.5	37.5	45.83	70.83	75	70.83	58.33	50
Mycoplasma bovis (48)	20.83	20.83	29.16	29.16	41.66	50	50	89.58	91.66	87.5	68.75	50
Enterococcus faecalis (38)	26.31	18.42	31.57	34.21	34.21	42.10	55.26	86.84	86.84	65.78	76.31	63.15
Klebsiella pneumoniae (94)	26.59	21.27	26.59	27.65	29.78	42.55	35.10	57.44	62.76	67.02	61.70	55.31
Pasteurella multocida (60)	26.66	16.66	31.66	40	35	48.33	60	95	100	80	78.33	66.66
Histophilus somni (18)	11.11	11.11	22.22	22.22	27.77	38.88	38.88	66.66	61.11	55.55	55.55	38.88
Bacteroides pyogenes (44)	22.72	15.90	27.27	36.36	36.36	38.63	47.72	75	84.09	63.63	65.90	50
Enterococcus faecium (56)	17.87	8.92	26.78	25	32.14	41.07	53.57	71.42	80.35	78.57	69.64	46.42
Clostridium perfringens (74)	29.72	24.32	25.67	28.37	28.37	43.24	55.40	83.78	86.48	82.43	75.67	54.05
Mycoplasma dispar (32)	40.62	28.12	34.37	46.87	40.62	56.25	62.50	93.75	93.75	93.75	87.50	68.75
Bacillus subtilis (76)	30.26	17.10	25	50	42.10	64.47	67.10	100	98.68	93.42	89.47	67.10
Pseudomonas taetrolens (52)	23.07	15.38	34.61	44.23	30.76	57.69	61.53	80.76	86.53	86.53	76.92	44.23
Mycoplasma alkalescens (36)	36.11	25	36.11	41.66	38.88	72.22	72.22	100	100	97.22	91.66	63.88
Bacillus obstructivus (30)	30	20	33.33	36.66	26.66	60	63.33	90	100	93.33	83.33	50
Streptococcus pneumoniae (98)	26.54	9.19	33.68	31.64	27.56	50	65.31	96.94	98.98	83.68	74.49	50
Staphylococcus aureus (102)	33.33	13.72	41.17	37.25	19.60	56.86	70.58	96.07	95.09	92.15	76.47	57.84
Micrococcus luteus (74)	25.67	17.56	31.08	43.24	27.02	45.94	68.91	82.43	90.54	89.18	90.54	74.32

Gen, Gentamicin; Sp, Spectinomycin; Ch, Chloramphenicol; Cip, Ciprofloxacin; Nor, Norfloxacin; Ery, Erythromycin; Lin, Lincomycin; Tet, Tetracycline; Pen, Penicillin; Cef, Cefoxitin; Ce, Cefoperazone; Am, amoxicillin.

As shown in Table II, *M. haemolytica* isolates exhibited >70% resistance against tetracycline, penicillin, and cefoxitin. However, these isolates showed the least resistance (4.16%) against spectinomycin. The isolates of *Mycoplasma bovis* were found highly resistant against penicillin (91.6%), while the least resistance (20.8%) was recorded against gentamycin and spectinomycin. The isolates of *Enterococcus faecalis* were found highly (86.8%) resistant against tetracycline and penicillin, followed by cefoperazone (76.3%). *K. pneumoniae* showed >60% resistance against penicillin, cefoxitin and cefoperazone. The isolates of *Pasteurella multocida* exhibited 100% and 95% resistance against penicillin and tetracycline, respectively. The isolates of *H. somni* were found highly resistant against tetracycline (66.6%), while the least resistance (11.1%) was recorded against gentamicin and spectinomycin. The isolates of *Bacteroides pyogenes* exhibited >60% resistance against four antimicrobials i.e., penicillin (84.1%), tetracycline (63.6%), cefoperazone (65.9%) and cefoxitin (63.6%); while least resistance was recorded against spectinomycin (15.9%). *Enterococcus faecium* isolates showed the highest resistance against penicillin (80.3%), followed by cefoxitin (78.5%), tetracycline (71.4%) and cefoperazone (69.6%). *Clostridium perfringens* isolates exhibited >80% resistance against tetracycline, penicillin and cefoxitin; while *Mycoplasma dispar* exhibited 93.7% resistance against these three antimicrobials. The isolates of *Bacillus subtilis* and *Mycoplasma alkalescens* were found fully resistant (100%) against tetracycline; while *Bacillus obstructivus* and *Mycoplasma alkalescens* were observed to 100% resistant against penicillin. *S. pneumoniae* exhibited 96.9% and 98.9% resistance against tetracycline and penicillin, respectively. The isolates of *Staphylococcus aureus* exhibited >90% resistance against tetracycline, penicillin and cefoxitin, while least resistance was recorded against spectinomycin (13.7%). The isolates of *Micrococcus luteus* showed 90.5% resistance against penicillin and cefoperazone; while 89.1% and 82.4% resistance were exhibited against cefoxitin and tetracycline, respectively.

The study of Anholt *et al*. (2017), reported that *P. multocida, T. pyogenes* and *M. haemolytica* were observed highly resistant to antimicrobials. The majority of the isolates exhibited resistance as high as 90.2%. Timsit *et al*. (2017) reported that *M. haemolytica* and *P. multocida* isolates of BRD exhibited > 70% resistance against oxytetracycline. These reports are in accordance with our current study, as we also recorded more than 70%

resistance for *P. multocida,* and *M. haemolytica* against the antibiotics tetracycline, penicillin and cefoxitin. De novo mutation is suspected of the high occurrence of resistance in bacterial isolates, which is consistent with the selection of multidrug-resistant mobile genetic elements or resistant pathogens that have them (DeDonder and Apley, 2015).

Welsh *et al*. (2004) published an 8-year study from 1994-2002 and reported a variable resistance (26-77%) for *M. haemolytica*, *H. somni* and *P. multocida* against tetracycline. Tetracycline was adopted for commercial use in 1978 and was recognized as a drug of choice till the end of the 20th century due to its broad antimicrobial spectrum, high availability and low cost (Gasparrini *et al*., 2020). At present, its' synergistic action and efficacy in localized infections is acceptable, however for systemic infections particularly those caused by Gram-negative organisms efficacy is highly compromised due to significant antimicrobial resistance developed in bacterial pathogens, human and animal commensals and environmental microbes (Chopra and Roberts, 2001). Ribosome protection and efflux are the main resistance mechanisms of bacterial organisms against tetracyclines (Gasparrini *et al*., 2020).

Streptococcus pneumoniae is known to colonize the nasopharynx of humans and animals, however, they were isolated commonly as a pathogenic isolate in fibrinous pneumonia and bronchopneumonia. Due to the high occurrence of *Streptococcus* in the environment and commensal microbiome, and their exposure to over-use of antimicrobials, critical antimicrobial resistance is been reported in animal and human studies (Hayes *et al*., 2020). In the current study *Streptococcus pneumoniae* exhibited ≥ 50% resistance against seven (out of twelve) tested antimicrobials which is also an alarming sign for clinicians to treat clinical cases of respiratory infections.

Mycoplasma is wall-less bacteria that are reported as a part of normal microflora in the respiratory and urogenital tract. They also cause mastitis frequently in dairy animals (Saif *et al*., 2022). In the current study, three species of *Mycoplasma viz*., *M. bovis, M. dispar* and *M. alkalescens* were isolated from respiratory tract samples. All these were observed highly resistant (50-100%) against erythromycin, lincomycin, tetracycline, penicillin, cefoxitin, cefoperazone, and amoxicillin. Being a wall-less bacteria, *Mycoplasma* is intrinsically resistant to cell-wall-targeting antimicrobials. In addition, recent literature has shown that they have established resistance against several other antibiotics like sulfonamide, nalidixic acid, rifampicin, polymixins, trimethoprim and rifampicin (Gautier-Bouchardon, 2018). Moreover, some other antibiotics like macrolides, tetracyclines, lincosamides, tiamulin and spectinomycin were observed as mycoplasmastatic instead of mycoplasmacidal action (Kleven and Anderson, 1971).

CONCLUSION

From the results, it could be concluded that *Staph. aureus*, followed by *Streptococcus pneumoniae* and *Klebsiella pneumoniae* were the most prevalent bacterial pathogens in the respiratory tract samples of cattle. Numerous bacterial isolates were observed more than 70% resistant against many antimicrobials. The antimicrobial spectrum results whistle an alarming situation to regulate the non-judicial use of antimicrobials.

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IRB approval

The Institutional Review Board (IRB) Approval was granted by the Animal Ethics Committee of Shihezi University (Approval No. A2022-14403).

Ethical statement

Animal handling during sampling was carried out in line with international ethical standard and IRB guidelines.

Statement of conflict of interest

The authors declared no conflict of interest.

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