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Molecular epidemiology and characterization of antibiotic resistance of *Pasteurella multocida* isolated from livestock population of Punjab, Pakistan

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ABSTRACT

Haemorrhagic septicaemia (HS) is an acute and life-threatening infection of livestock population caused by *Pasteurella multocida* (*P. multocida*), responsible for huge mortality, morbidity and production losses. The increase in antibiotic resistance is a growing concern, posing a significant threat to animals and public health. There is limited data on *P. multocida* disease burden, serotypes, antibiotic susceptibility, and resistance gene profiles in Pakistan. In the current study, 1017 nasal swabs from haemorrhagic septicaemic cattle and buffaloes were collected to isolate *P. multocida* through microbiological and molecular methods. Susceptibility against commonly used antibiotics was performed and antibiotic resistance genes were evaluated. A prevalence rate of 7.57% was found, where buffaloes were more prone to infection (8.3%) as compared to cows (6.7%). Molecular and sequence analysis confirmed *P. multocida* isolates in 94.8% (73/77) of samples. Capsular typing revealed all isolates belong to serotype B. Antibiogram analysis showed that enrofloxacin 85.7% (66/77) and ceftiofur 56/77 (72.7%) were the most effective antibiotics. The highest resistance was observed against trimethoprim/sulfamethoxazole 54/77 (70.1%), followed by erythromycin 52/77 (67.5%). Most of the isolates (31.5% (23/73)) carried β-lactamase resistance genes (bla_{TEM} $n = 10$, bla_{ROB-1} $n = 6$, bla_{OXA-2} $n = 5$, bla_{NDM} $n = 2$) followed by trimethoprim/sulfamethoxazole (*sul2*) resistance genes (26% (19/73)). The current study indicates that HS is consistently circulating among the animal population in Punjab, Pakistan. The current scenario of higher resistance in *P. multocida* needs continuous surveillance of the infection and mass awareness programs about the non-prescribed and excessive use of antibiotics in the animal sector.

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1. Introduction

Hemorrhagic septicaemia (HS) is a significant acute infection in the livestock population. It is an economically important disease, responsible for huge mortality, morbidity and production losses [1]. HS is responsible for an approximate annual loss of billions of Pakistani rupees.

Capsular serotypes B and E of *P. multocida* are responsible for causing HS in animals [2]. It is considered an opportunistic pathogen and is generally observed in the upper part of the respiratory tract of wild and domestic animals. Most of the infections caused by *P. multocida* [3] are fowl cholera in poultry, atrophic rhinitis in swine, pneumonia in rabbits, small ruminants and pigs, as well as acute septicaemia in buffaloes and cattle [4]. Pakistan is an agricultural country where 58.7% of animal deaths were recorded due to various endemic infections, including blackleg, anthrax, rinderpest, HS and foot and mouth disease (FMD) [5]. A high death toll and high financial losses, i.e. 77% and 23%, respectively, were observed due to HS as compared to Foot and Mouth Disease [6,7].

Carter system classifies *P. multocida* into five different serotypes based on capsular antigens, including A, B, D, E and F. Based on somatic antigens performed through gel diffusion precipitin method according to the Heddleston system, there are 16 serotypes (1 to 16) [8,9]. However, HS in livestock is caused by two serotypes, including E2 (African Type) and B2 (Asian Type) [10]. Various antibiotics, including neomycin, streptomycin, chloramphenicol, tetracycline, ampicillin, or penicillin, are effective in treating various infections, including HS, during the early stage of infections [6,11].

The burden of non-prescribed antibiotics in livestock populations leads to the enhancement of selective pressure on the micro-organisms to gain and express genes related to antibiotic resistance, ultimately increasing the number of resistant isolates [12]. Global data suggest that during the preceding few years, an increase

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has been observed in the incidence of multidrug-resistant (MDR) isolates [13], responsible for limiting therapeutic options for infectious diseases in livestock populations. Furthermore, zoonotic bacterial pathogens show more antimicrobial resistance, which is a serious concern to the human population as they directly pass this resistance from food-producing animals to humans [14]. This phenomenon enforces international and national institutions to invigilate, systematize and control the excessive use of antibiotics [15,16]). Henceforth, the World Health Organization (WHO) and the World Organization for Animal Health (WOAH) have proposed a list of few antibiotics that are critically important and can be used for human and veterinary medicinal practices [17,18].

The proper control and prevention of HS require the use of effective antibiotics and a detailed survey in the respective geographical areas where the infection is prevalent [19]. The scarcity of data on surveillance of antibiotic resistance profiling, molecular epidemiology, variation in capsular types of *P. multocida* is responsible for huge economic losses in livestock population of Pakistan. Therefore, this study was designed to evaluate molecular epidemiology, genetic characterization, and the pattern of antibiotic resistance in *P. multocida* isolated from the livestock population in Punjab, Pakistan.

2. Methodology

2.1. Study site demographic and sampling information

Pakistan is an agricultural and developing country with five provinces. Punjab is located in the middle of the country with an area of 205,344 square kilometres, and it shares borders with all other provinces and the capital, Islamabad. The province of Punjab is located at the latitude of 31.170406° N and longitude of 72.709716° E, respectively. It is the homeland of five rivers with nine administrative divisions and 36 districts. It includes agricultural areas with hot climatic summer conditions with various livestock upraising [20] (Figure 1). Most of Pakistan's livestock is present in Punjab, with 65% and 49% of the buffalo and cow population, respectively. A total of 1017 nasal swabs were collected from suspected buffaloes and cows from almost all districts of Punjab (Supplementary table 1).

3. Ethical approval

This study was approved by the Ethical Committee of Pir Mehar Ali Shah (PMAS) Arid Agriculture University, Rawalpindi, Punjab, Pakistan (Ref: PMAS-ARID-1231)19).

4. Sample collection, and identification

A total of 1017 ($n = 507$ cattle and $n = 510$ buffaloes) nasal swabs were collected throughout the year from animals with average age of 2.1 years representing signs of shallow and rapid breathing, moist cough, nasal discharge, loss of appetite, fever ≥41.5°C, and depression. The male-to-female ratio among cattle was 56.96, and in buffaloes, it was calculated as 26.55. Nasal swabs ($n = 10$) from healthy animals were also collected to run as a control. Brain heart infusion broth (Difco, New Jersey, USA) was used to transport nasal swabs. The samples were properly labelled, followed by packing, placed in a cooled icebox, and aseptically transferred to the Antimicrobial Resistance Laboratory, PMAS-ARID Agriculture University, Rawalpindi, Pakistan. The

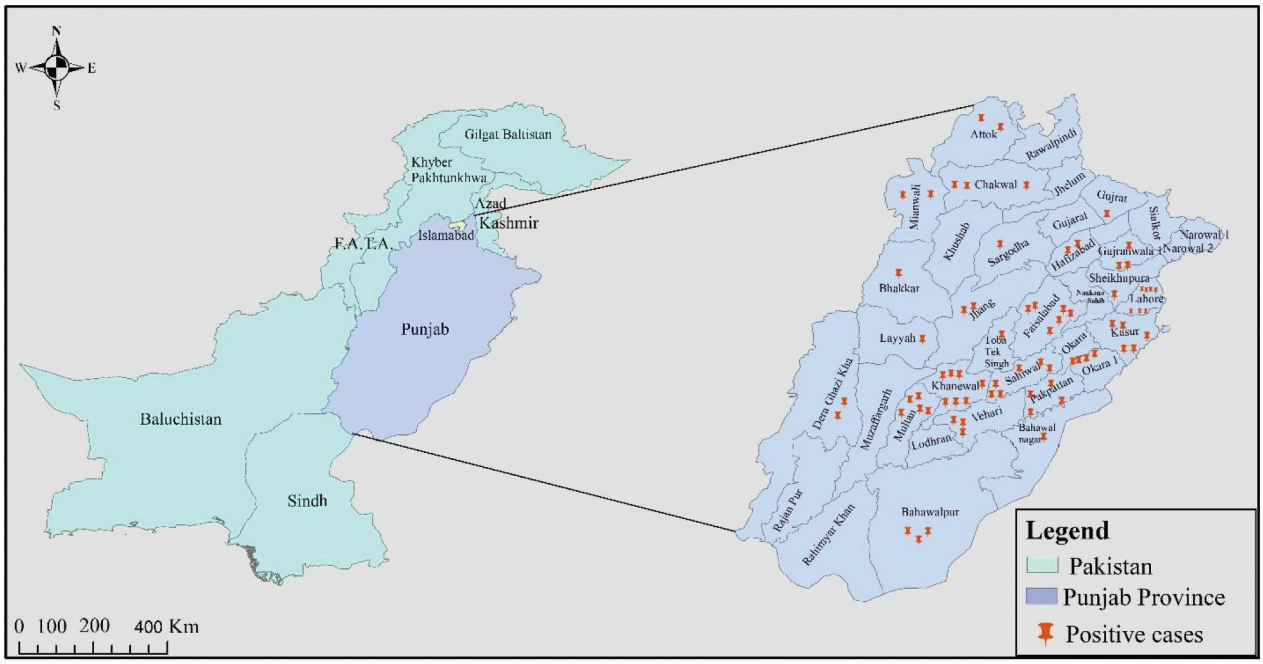

Figure 1. Map of the Punjab Pakistan showing the studied districts.

samples were kept at 4°C while being processed. Enrichment broth containing brain heart infusion, 5% sheep blood (defibrinated) (Remel, San Diego, CA, USA), amphotericin B (5.0 µg/mL), potassium tellurite (0.0125 µg/mL) (Sigma Chemical Co., St. Louis, MO, USA), and gentamicin (0.5 µg/mL) (Gibco, Grand Island, NY, USA) was prepared, and one mL of sample from the transported vile was transferred into it with the help of a sterile pipette to begin the subsequent tests. Like the enrichment broth medium, the medium's ingredients, concentration and pH were modified. The pH was maintained at 10.0 ± 0.1 by using sterile 1 N NaOH. After preparing the selective agar medium, streaking was done by a loop from the tubes, which showed turbidity after incubation at 37°C for 16 hours in 5–10% CO_2. This process allowed for isolating *P. multocida* colonies on the selective agar medium [21]. Microbiological confirmation of presumptive colonies of *P. multocida* (3–5) was obtained through microscopic examination and biochemical tests. Besides no growth on MacConkey agar, the colonies grew on a selective medium and appeared smooth, mucoid, and sticky. Gram staining showed that *P. multocida* was Gram-negative, round-ended, small, short rods, or oval-shaped. Biochemical tests showed that this bacterium could ferment fructose, mannose, sucrose, and glucose sugar and showed positive results in the catalase, nitrate reduction, indole production, and H_2S production tests. Further confirmation of positive colonies was done through the Biolog GP2 and GN2 micro-plates and the Biolog Microlog 3.70 software and database (Biolog, Hayward, CA), as mentioned earlier [22]. The strains of *E. coli* ATCC 11,775 and *P. multocida* ATCC 43,137 were used as negative and positive controls, respectively.

5. Molecular confirmation and characterization of *P. multocida*

The extraction of DNA was done using the QIAamp kit (Qiagen GmbH – Germany) as recommended in the instruction manual. The molecular confirmation of the 77 *P. multocida* isolates was performed using a primer set for the *Kmt1* gene, synthesizing an integral part of the membrane, as reported earlier [23] (Table 1). PCR-amplified products were selected randomly and then sequenced with the help of forward and reverse primers in both directions (Macrogen Inc., Seoul, South Korea). Sequencing was performed via the Chromas (Technelysium Pty Ltd., Queensland, Australia) software, and the sequences obtained were subjected to alignment and assembly by DNASIS MAX (version 3.0; Hitachi, Yokohama, Japan) software. Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov) for phylogenetic similarity in the database (GenBank) was searched randomly [25]. The final sequence of each sample was subjected to comparative alignment with the reference sequence. The phylogenetic tree was generated using the Taimura-Nei method with 1000 bootstrap tests for genotyping of *P. multocida* through MEGA X software (www.megasoftware.net) based on the study of [26].

6. Capsular serotyping of *P. multocida* isolates

Capsular serotyping was performed to check the prominent serotypes among *P. multocida* isolates. A multiplex PCR technique was used for serotyping. The primers used for Capsular typing, and their amplicon size are mentioned in Table 1. Two different codes (2 and 1) were used for positive control isolates, which represented capsular serotypes B and E, respectively. The positive control isolates were obtained from Veterinary Research Institute (VRI) Lahore.

7. Antibiogram analysis of obtained *P. multocida* isolates

Antibiogram analysis of each isolate of *P. multocida* was carried out using a panel of eight antibiotic discs, including erythromycin, florfenicol, ampicillin, tetracycline, ceftiofur, enrofloxacin, trimethoprim/sulfamethoxazole, and streptomycin (Oxoid, San Diego, CA, USA). Antibiotics were selected based on Clinical and Laboratory Standard Institute (CLSI) VET01S (5th ed:2020) guidelines and their medical importance [17]. Kirby Bauer's disk diffusion method was used to check the antibiogram analysis, and the results were interpreted through the guidelines provided by the Clinical and Laboratory Standard Institute (CLSI) VET01S (5th ed:2020), M45 (3rd ed:2015), and the VET01- A4 (4th ed: 2013). This study used the *P. multocida* ATCC 43,137 strain as a positive control.

8. Molecular detection of antibiotic resistance genes

All confirmed isolates were screened for the presence of macrolide – lincosamide – streptogramin B methylases *erm(A)*, *erm(B)*, *erm(C)*, extended-spectrum β-lactamases (ESBLs) genes including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{OXA}, bla_{ROB}, bla_{NDM}, sulphonamide resistant dihydropteroate synthase *sul2*gene, florfenicol/chloramphenicol resistant gene *floR*, streptomycin-resistant gene *strA* and tetracycline-resistant gene *tetA*, *tetB,* and *tetH* (Table 1). The PCR reaction volume of 25 µL, comprising of ready-to-use master mix (12.5 µL), upstream and downstream primers (0.5 µL) each (50 pmol/µL) (Nojihigashi, Kusatsu, Shiga, Japan), RNase-free water (9.5 µL), and bacterial genomic DNA (2 µL) (100 ng/µL). The isolate coded 14 was used as the positive control for macrolide genes, the extended-spectrum β lactamase and the ampicillin resistance genes. The isolate coded 15 was used as the positive control for the sulphonamide-resistant dihydropteroate synthase, and florfenicol/chloramphenicol resistance genes. Additionally, to confirm

Table 1. Primer sequences, estimated amplicon size, and annealing temperature.

Gene Name	Primer sequence (5′-3′)	Amplicon size	Annealing Temp	Purpose	Reference
KMT1	F;ATCCGCTATTTACCCAGTGG R;GCTGTAAACGAACTCGCCAC	460 bp	55°C	Primers were used for confirmation of isolates targeted to an essential component of the membrane.	[24]
Capsular Typing					
Capsular type A	CAPA-F-CATTTATCCAAGCTCCACC CAPA-R- GCCCGAGAGTTTCAATCC	1140	55°C	Molecular capsular typing	[24]
Capsular Type B	CAPB-F-CATTTATCCAAGCTCCACC CAPB-R-GCCCGAGAGTTTCAATCC	760	55°C		
Capsular type D	CAPD-F-TTACAAAAGAAAGACTAGGAGCCC CAPD-R-CATCTACCCACTCAACCATATCAG	657	55°C		
Capsular type E	CAPE-F-TCCGCAGAAAATTATTGACTC CAPE-R-GCTTGCTGCTTGATTTTGTC	511	55°C		
Capsular type F	CAPF-F-AATCGGAGAACGCAGAAATCAG CAPF-R-TTCCGCCGTCAATTACTCTG	851	55°C		
Antimicrobial Resistance Genes (ARGs)					
erm(X)	F-GGCGGCGAGCGACTTCC R-GGCGGCGAGCGACTTCC	724	60°C	lincosamide – streptogramin B methylases	
erm(A)	F-TCTAAAAAGCATGTAAAAGAA R-CTTCGATAGTTTATTAATAGT	1156	51°C		
erm (B)	F-GAAAAGGTACTCAACCAAATA R-AGTAACGGTACTTAAATTGTTTAC	639	52°C		
flor	F-ATGGCAGGCGATATTCATTA R-AAACGGGTTGTCACGATCAT	320	55°C	Florfenicol/ chloramphenicol resistance genes	
sul2	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	284	70°C	sulfonamide-resistant dihydropteroate synthase	
bla_{TEM}	F-GCGGAACCCCTATTTG R-ACCAATGCTTAATCAGTGAG	757	55°C	Ampicillin resistant Extended spectrum β-lactamase gene	
bla_{CTXM}	F-ATGTGCAGYACCAGTAARGTKATGGC R-TGGGTRAARTARGTSACCAGAAYSAGCGG	593	60°C	cefotaximase Extended spectrum β-lactamase	
bla_{SHV}	F-TTATCTCCCTGTTAGCCACC R-GATTTGCTGATTTCGCTCGG	790	60°C	β-lactamase-sulf-hydryl variable active	
bla_{OXA}	F-GCGTGGTTAAGGATGAACAC R-CATCAAGTTCAACCCAACCG	438	55°C	Oxacillin type β-lactamase	
bla_{NDM}	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	621	55°C	New Delhi metallo- β-lactamase	
*tet*A	F-GTA ATT CTG AGC ACT GTC GC R-CTG CCT GGA CAA CAT TGT TT	1075	62 °C		
*tet*B	F-CCT TAT CAT GCC AGT CTT GC R-ACT GCC GTT TTT TTC GCC	774	50 °C		
*tet*H	F-ATACTGCTGATCACCGTATAGATG R-TCCCAATAAGCGACGC	1175	50 °C		

the positive or negative results, PCR was done in triplicate.

9. Statistical analysis

The entire collected data was transferred to SPSS version 23.0.2 (Statistical Package for the Social Sciences). A chi-square test (x^2) was performed, keeping a 95% confidence interval. A p value of less 0.05 was considered significant.

10. Results

A total of 77/1017 (7.57%) samples were positive for HS/*P. multocida* phenotypically. The animal-wise distribution showed that buffaloes were more prone to infection with a prevalence of 43/510 (8.3%) than cows at 34/507 (6.7%). The gender-wise distribution revealed that females were more infected with HS 61/77 (79.2%). Age-wise distributions showed that animals of age 2-3 yrs were significantly associated with HS ($p < 0.05$), as shown in Table 2.

11. District-wise distribution of the cases

The current study reported that most of the cases were from districts Lahore, Faisalabad, and Khanewal, 7.7% each. The lowest prevalence was 1.2% each from Layyah, Bakkar, Toba Tek Singh, Sheikhupura, Bahawalnagar, and Gujrat districts, as shown in Figures 2 and 1.

12. Antibiogram analysis of *P. multocida*

The antibiotic susceptibility showed that enrofloxacin 66/77 (85.7%) and ceftiofur 56/77 (72.7%) were the most effective antibiotics. In comparison, the highest resistance was observed against trimethoprim/sulfamethoxazole 54/77 (70.1%), followed by erythromycin 52/77 (67.5%), as shown in Figure 3.

Table 2. Prevalence of *P. multocida* among animals of different ages and gender.

Characteristics	Female n/N (%)	Male n/N (%)	Total (%)	P-value	Chi-square
Animal and Gender Wise Distribution					
Cows	25/34 (73.5%)	09/34 (26.4%)	34/507 (6.7%)	0.18	4.778
Buffaloes	36/43 (83.7%)	07/43 (16.2%)	43/510 (8.3%)		
	Cows' n/N (%)	Buffaloes' n/N (%)	Total (%)	P-value	Chi-square
Animal and Age-Wise Distribution					
Age group 1 (below 2 yr) (43.96%)	09/34 (26.4%)	12/43 (27.9%)	21/77 (27.2%)	0.03	6.893
Age group 2 (2–3 yr) (38.33%)	18/34 (52.9%)	19/43 (44.1%)	37/77 (48.0%)		
Age group 3 (above 3 yr) (17.17%)	07/34 (20.5%)	12/43 (27.9%)	19/77 (24.6%)		

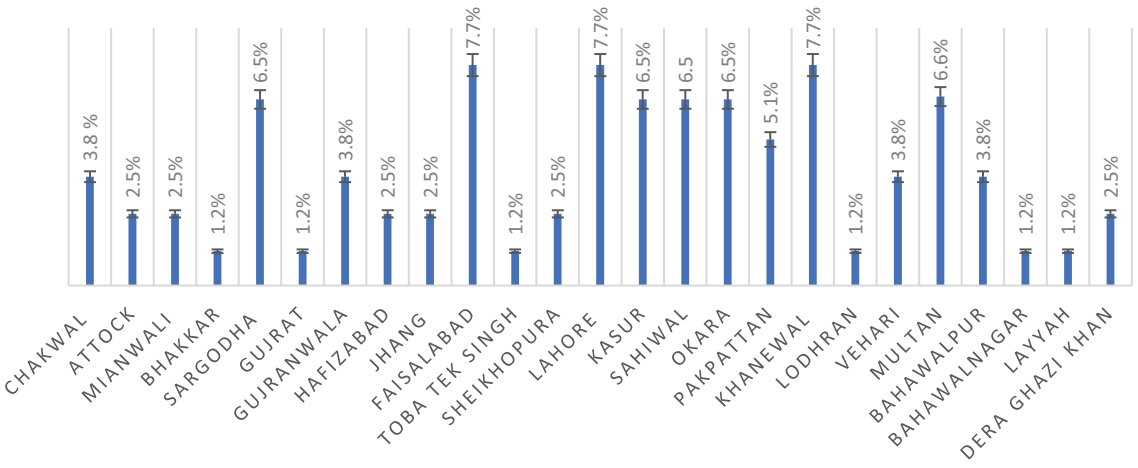

Figure 2. District-wise distribution of the hs-positive cases.

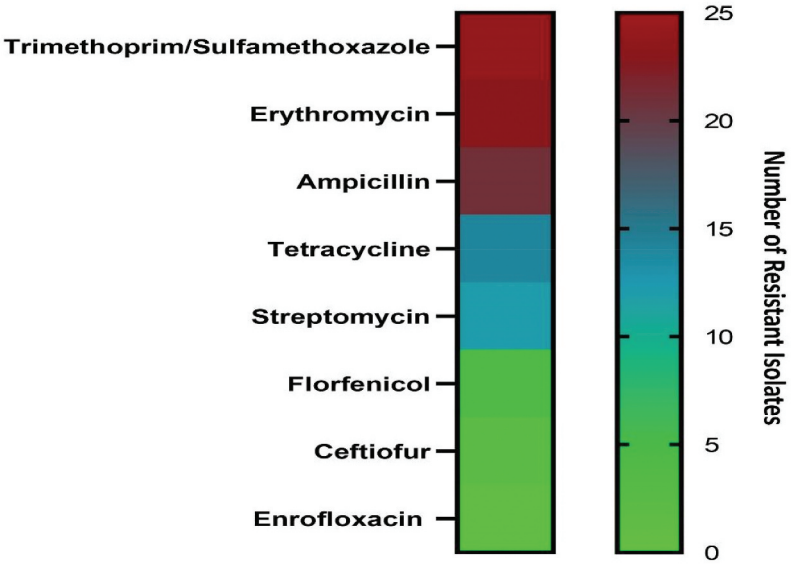

Figure 3. Antibiotic resistance profile of *P. multocida* isolates.

13. Molecular characterization of *P. multocida* isolates

A total of 73/77 (94.8%) were found to be positive for *kmt1* gene (Supplementary Figure 1), among which 30 isolates were selected for sequencing, which confirmed *P. multocida* as shown in the phylogenetic tree (Figure 4).

14. Genotypic characterization of *P. multocida* isolates

A multiplex PCR was done using the primers mentioned in Table 1, and a region of 760 bp was identified for all isolates (Figure 1 Supplementary), indicating that all the isolates belonged to serotype B.

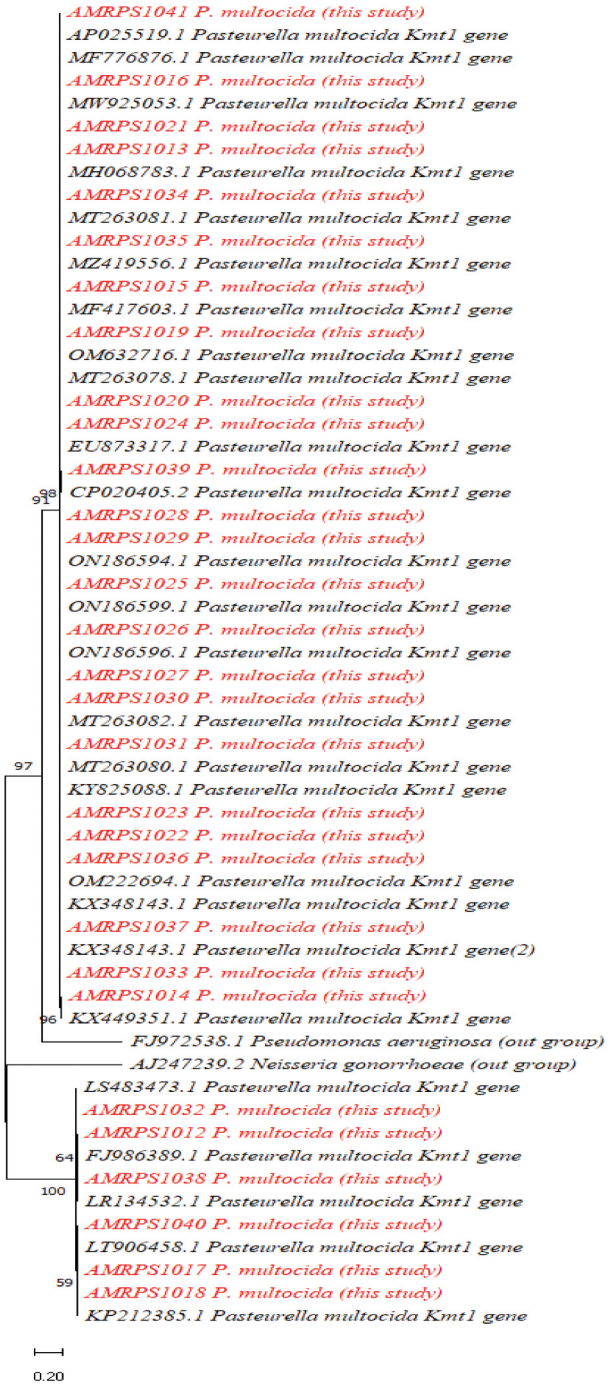

Figure 4. The phylogenetic tree was constructed using Maximum likelihood methods based on nucleotide sequences of the Kmt1 gene showing *Pasteurella multocida* isolates obtained from the livestock population in Punjab, Pakistan.

15. Characterization of antibiotic resistance genes

The present study observed that β-lactamases resistance genes were more prevalent 23/73 (31.5%), including bla_{TEM} $n = 10$, $bla_{\text{ROB-1}}$ $n = 6$, $bla_{\text{OXA-2}}$ $n = 5$, bla_{NDM} $n = 2$, followed by trimethoprim/sulfamethoxazole resistance genes 19/73 (26%), erythromycin (*erm*) 16/73 (21.9%), tetracycline (*tet*) 10/73 (13.6%), streptomycin 09/73 (12.3%), and florfenicol 03/73 (4.1%), as shown in Figure 5.

16. Discussion

HS is an extremely complex and multifactorial infection affecting livestock populations. It is caused by *P. multocida*, a bacterium that infects the respiratory tract of susceptible animals (compromised immune system and stress condition). The current study reported the prevalence of *P. multocida* in 7.57% of symptomatic HS cattle and buffaloes in Punjab, Pakistan. Previous studies from Pakistan also reported *P. multocida* from livestock populations infected with HS [27–29]. Earlier studies from other countries also isolated *P. multocida* from HS symptomatic and non-symptomatic cattle. Furthermore, *P. multocida* and *M. haemolytica* were also found in several samples collected from the diseased animals [30], where *P. multocida* was more prevalent as compared to *M. haemolytica* [31]. The non-availability of an effective vaccine might be the cause of the high infection rate of *P. multocida* [32]. The molecular epidemiological study of haemorrhagic septicaemia in the livestock population of Pakistan will help in early detection of future outbreaks, the zoonotic potential of the disease, timely interventions, and development of strategies for better management of HS.

The PCR method was found to be quick and accurate method for the confirmation of *P. multocida* through a species-specific gene (*Kmt1*), and to check the capsular serotype as previously observed [33–37]). In the present study, all isolated *P. multocida* belonged to serotype B, like other studies reporting that serotype B was present in entire studied livestock animals [38–40]. These studies agree with the finding of *P. multocida* serotype B from haemorrhagic septicaemia-infected buffaloes and cattle from various tropical regions of the world [41]. However, infections in pigs are caused by capsular serotype D [23]; it might also be responsible for infections in cattle [42]. Serotype B is prevalent in most of Asia. The similarities and differences in the serotypes infecting cows and buffaloes might be due to multiple reasons. Animal trade of Pakistan with other neighbouring countries, as well as serotype E or B of *P. multocida*, could be colonized in a small number of healthy cattle and buffaloes, which can be shed during stress periods like humidity, high temperature, work stress, poor nutrition, and concurrent infection [43]. Identifying *P. multocida* strains circulating in the cattle population of Pakistan enables more focused and efficient control measures, including the production of vaccines, that are more effective since they are matched to the local strains.

In this work, most of the isolates showed sensitivity to enrofloxacin, followed by ceftiofur, and a high level of resistance was observed against trimethoprim/sulfamethoxazole fixed-dose combination (FDC), followed by erythromycin. Similar results of the highest

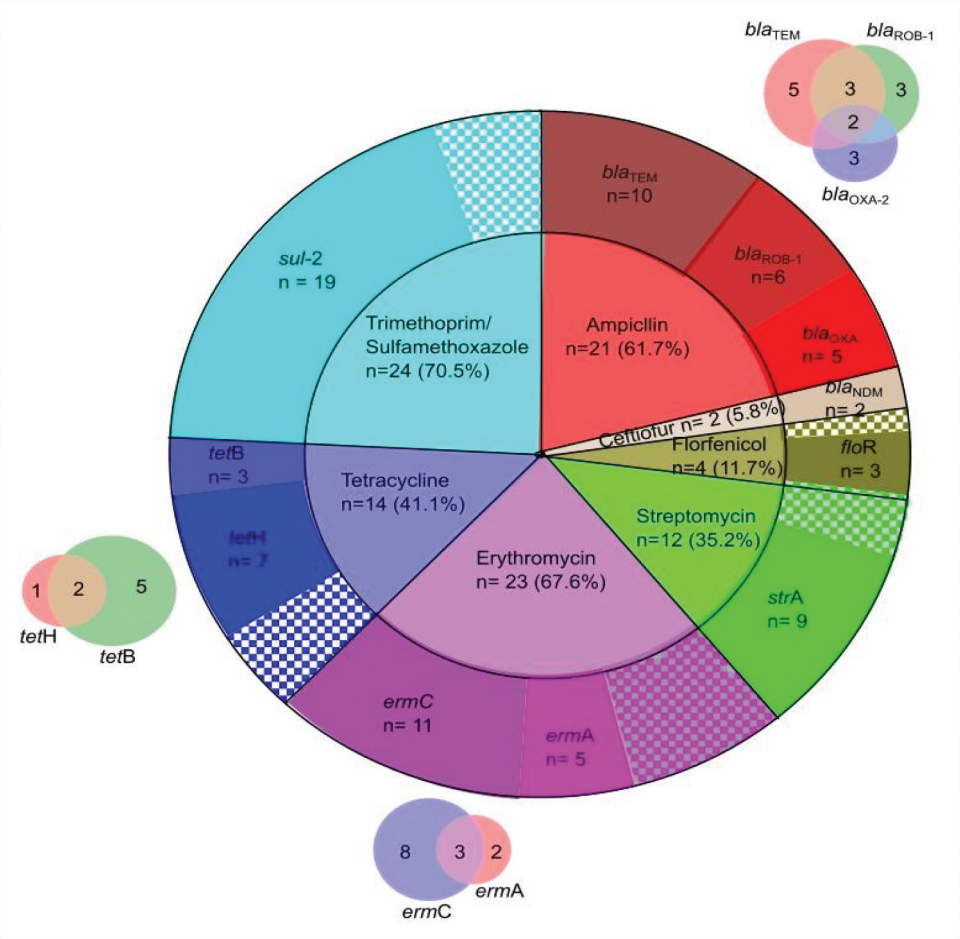

Figure 5. Phenotypic and genotypic resistance among *P. multocida* isolates.

sensitivity for enrofloxacin while resistance against erythromycin have been reported in an earlier study from Pakistan [44]. These antibiotic susceptibilities agreed with previously reported results [45](. Subsequently, 1% of Pasteurella isolates from Canada and the USA exhibit resistance, ceftiofur is still potentially very effective [46,47] [48,49]. The highest resistance against trimethoprim/sulfamethoxazole antibiotic [33,47] and erythromycin [50,51, 52] has been consistently reported in previous studies. Recently, the increasing resistance trends of *Pasteurella* spp. against several routinely used antibiotics [46,48,49,53] might pressurize us towards increased use of ceftiofur antibiotics against extended-spectrum β-lactamase (ESBLs) producing *Enterobacteriaceae* and Bovine Respiratory Disease [54]. Consequently, the excess use of ceftiofur antibiotics in husbandry practices results in more cases of cephalosporins cross-resistance, negatively affecting human therapies. The currently observed resistance against trimethoprim/sulfamethoxazole and erythromycin is high. This could be due to non-prescribed and excessive use of antibiotics in dairy farms and poultry farms as a feed additive and growth promoter [55]. Furthermore, the antibiotic-resistant strains of *P. multocida* might have the ability to inherit resistance genes through horizontal gene transfer from other bacteria of the same class [56]. The overuse use of antibiotics in livestock populations inserts selective pressure on the gastrointestinal microbial flora, which leads to the acquisition of antimicrobial-resistant genes containing bacterial community in the bovine digestive system [57]. Through horizontal gene transfer, the antimicrobial-resistant genes could be acquired and transmitted among beneficial and opportunistic bacteria within the digestive tract-associated microbial community. In the same manner, the transfer of resistance genes from one bacterium to another will positively impact bovine-associated human pathogens, becoming an important public health concern [57]. Humans may come into direct contact with animals that are colonized or afflicted with antibiotic-resistant bacteria, or they may come into touch with biological materials including blood, urine, excrement, saliva, and semen as well as indirectly through the food chain by consuming contaminated food [58].

Antibiotic resistance is increasing day by day, and it is causing a great challenge to public health [59]. During the last decades, ARGs have been identified in every matrix including the natural environment [60–62], engineered [63–66], and clinical [67,68]

habitats. One of the main drivers of ARG dissemination are anthropogenic activities, which also include the clinical use of antibiotics [65,69,70]. The current study observed the highest percentage of β-lactam resistance genes (bla_{TEM}, bla_{ROB-1}, bla_{OXA-2}, and bla_{NDM}) among *P. multocida* isolates as found in five of the β-lactamase (*bla*) genes among *Pasteurellaceae* including bla_{CMY-2}, bla_{PSE-1}, bla_{OXA-2}, bla_{ROB-1}, and bla_{TEM-1} [38]. Several reports observed bla_{CTX-M} and $bla_{CTX-M-1}$ in *P. multocida* isolate from cattle and humans [38] and bla_{ROB-1} [71,72] from diseased rabbits and diseased chicken, respectively. It is also reported that the bla_{TEM-1} gene is present in human infecting pathogenic bacteria [73] and cattle pathogens [74]. Nevertheless, other papers described the coexistence of bla_{OXA-2} [47], bla_{ROB-1} in *M. haemolytica*, and the latest with bla_{ROB-2} (Kadlec et al., 2018). That is, it reduces the concentration of the active elements in the β-lactam antibiotics, produces low-affinity PBPs or acquires a β-lactamase enzyme to enable *Pasteurellaceae* to become resistant [74]. Several other mechanisms, like the expression of multidrug efflux pump, which expel β-lactams from bacterial cells and decreased outer membrane permeability, are rarely observed in *Pasteurellaceae* [74].

ARGs associated with macrolide resistance, like *erm* (42), *msr* (E), and *mph* (E), are less frequently reported, besides the common observation of phenotypic resistance against macrolide among both *M. haemolytica* and *P. multocida* [47]. The 23S rRNA gene has several mutations, which in turn can cause high levels of resistance in *M. haemolytica* and *P. multocida* against macrolides [75]. The presence of clindamycin resistance in the absence of erm suggests that there is another mechanism that contributes to resistance against clindamycin [47]. The current study supports the earlier reports of erythromycin-resistant genes (*erm*A, *erm*C) in *P. multocida* isolates from bovine origin. There are two genes involved in azithromycin and erythromycin resistance, which are *remand msr*(E)-*mph*(E) [47,76,77]). Ujvári et al. and Rose et al. reported the presence of resistance isolates without the presence of any genes that is (genotype −ve/phenotype+ve) [77,78]. The dissemination of sensitive bacteria containing the resistance genes (genotype -ve/phenotype +ve) indicates that they were silent and that there are other modes of resistance, as pointed out by another study [79].

The tetracycline-resistant genes (*tet*A, *tet*B, *tet*H) have been found among *P. multocida* isolates from the bovine origin, which is consistent with the presence of *tet* (H), as reported by Klima et al. [47]. Samples of *P. multocida* collected from the USA have shown the presence of *tet* (M) and *tet* (B) [59]. The *tet* (G) was also reported from Taiwan [80]. Most of the sulphamethazine- and trimethoprim/sulfamethoxazole-resistant isolates have the *sul2* gene in their genome [47]. In this study, we found the trimethoprim/sulfamethoxazole-resistant genes (Sul-2) among *P. multocida* isolates from bovine origin. In this present study, the chloramphenicol (*florR)* gene conferring resistance was detected in *P. multocida* isolates originating from bovines, similar to the previous study conducted on *P. multocida* isolates obtained from pigs from Vietnam [81]. In 1996, the fish pathogen *Photobacterium damselae* subsp. *Piscicida* was examined to observe having *floR* gene on its plasmid [82]. The current study observed that there is streptomycin-resistant gene (str) in *P. multocida* isolates from bovine origin, as reported by several reports [83,84]. Similarly, tetracycline (*tetR-tet*(H), streptomycin (*strA-strB*), and sulphonamide (*sul2*) resistance were reported from *P. multocida* in an earlier study [84]. Chloramphenicol (Cm), streptomycin (Str), or sulphonamides (Sul) indicate the presence of four variants of plasmids that produce resistance to sulfamethoxazole, streptomycin and chloramphenicol. Multidrug-resistance gene clusters are particularly significant because they are the reasons that cause resistance against a group of antimicrobials [83,85]. The presence of clustered ARGs on plasmids could be easily transmitted among various genera, species, and strains. Even without direct selective pressure, disseminating a plasmid containing a multi-resistance gene cluster carries the risk of co-selection and the constant presence of resistance genes. Earlier reports also revealed the widespread prevalence of *sul2-strA*-carrying plasmids in gram-negative bacteria [83,86,87] and that they are also able to accept other resistance genes as well, such as *catA3* [83,85] and *dfrA14* [88], to form new resistance gene cluster. The environment serves as a reservoir for antimicrobial resistance (AMR) and is crucial to the spread of antimicrobial resistance genes (ARGs). This can occur through direct means such as the use of antibiotics in healthcare, agriculture, and livestock, or indirectly through the discharge of antibiotic residues from various home settings [89,90]. Detection of antimicrobial resistance genes in *P. multocida* isolates retrieved from livestock in Punjab, Pakistan can guide treatment procedures and ensure antibiotic use with caution. This is crucial for controlling HS in a way that prevents the emergence and dissemination of resistant strains.

17. Conclusion

Conclusively, the prevalence of *P. multocida* was 7.57% among symptomatic HS cattle and buffaloes. All isolates belonged to serotype B; the most effective

antibiotic was enrofloxacin, and the least effective one was trimethoprim/sulfamethoxazole. The prevalence of β-lactam resistance genes (bla_{TEM}, bla_{ROB-1}, bla_{OXA-2}, bla_{NDM}), trimethoprim/sulfamethoxazole-resistance genes (*Sul-2*), macrolide-resistance genes (*erm* A&C), and tetracycline-resistance genes (*tet* H&B) among *P. multocida* isolates were observed in this study. The prevalence of *P. multocida* serotype B, ARGs and associated resistance will guide practitioners in choosing the appropriate treatment options. It will also provide a base for policymakers to define the guidelines for properly using antibiotics in livestock populations. The study's limitations include underreporting of the disease, challenges related to farmer participation, difficulties in sample collection, limited funding opportunities and the resources and instruments needed for in-depth genomics and proteomics studies.

18. Recommendations

To overcome antibiotic resistance and reduce reliance on antibiotics, alternative farming practices are highly recommended. Similarly, large-scale longitudinal studies in other parts of Pakistan will help in better understanding the changes in the antibiotic resistance pattern. Moreover, mass awareness programs addressing the irrational use of antibiotics, whole genome sequencing, virulence-related gene identification, and the determination of different antigenic structures for vaccine development are needed to tackle the most pressing issue of AMR in the livestock population of Pakistan

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Disclosure statement

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Authors' contributions

MY and RZ designed the study. SA and MHAT collected data. SA performed the lab work, analysed the data and wrote the manuscript. MY, MU, and RZ critically evaluated and drafted the final manuscript. All authors approved the final draft.

Statement of Data Availability

The data represented in this study is available, which might be shared upon reasonable request to the Corresponding Author.

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