



Full Length Article

Phenotypic Characterization, Molecular Identification and *In Vitro* Susceptibility of Bubaline *Pasteurella multocida* to Ceftiofur Hydrochloride

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Abstract

Clinical isolate of *Pasteurella (P.) multocida* recovered from a naturally infected water buffalo (*Bubalus bubalis*) and a reference strain (serotype B:2, strain P₅₂) were identified on the basis of culture characteristics, colonial morphology and biochemical reactions. Following the pathogenicity assessment by mice lethality test, the organisms were confirmed as *P. multocida* B:2 through specie-specific and type-specific polymerase chain reactions. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill kinetics of ceftiofur hydrochloride were established against *P. multocida* using broth microdilution assay. The MIC and MBC of ceftiofur hydrochloride were estimated as 0.15 µg/mL and 0.30 µg/mL, respectively against the reference isolate. Whereas, an MIC of 0.30 µg/mL and MBC of 0.62 µg/mL were recorded upon the exposure of clinical isolate to ceftiofur hydrochloride. The mean MIC and MBC values of ceftiofur hydrochloride were 0.22 µg/mL and 0.45 µg/mL, respectively. Overall, time and ceftiofur concentration significantly affected the bacterial density. Nevertheless, the bacterial count remained comparable during 0–6 h followed by progressive bacterial killing at time points of 8, 12 and 24 h. Contrary to ceftiofur concentration below the MIC, concentrations ≥ MIC substantially declined the bacterial colony count. Taken together, the estimated MIC and MBC values along-with corresponding time-kill data rationalized the adequate *in vitro* efficacy and time-dependent bactericidal activity of ceftiofur hydrochloride against *P. multocida*. © 2019 Friends Science Publishers

Keywords: *Pasteurella multocida*; Ceftiofur; MIC; MBC; Time-kill kinetics

Introduction

Pasteurella multocida, the Gram-negative, bacterial pathogen is known as the etiological agent of several economically important diseases in many animal species (Boyce and Adler, 2006; Gull *et al.*, 2015; Han *et al.*, 2018). Hemorrhagic septicemia (HS) is an endemic, acute and fatal disease of domestic and wild mammals caused by *P. multocida* (Shivachandra *et al.*, 2011). Affected animals, particularly the young calves of cattle and buffaloes usually suffer from huge morbidity and mortality (Khan *et al.*, 2011). Furthermore, the water buffaloes are comparatively more vulnerable to HS than cattle (Alwis, 1999). HS outbreaks frequently occur in tropical regions during rainy season owing to lack of proper vaccination coupled with increased environmental temperature and high humidity (Farooq *et al.*, 2011). Treatment is anticipated by parenteral administration of effective antimicrobial drugs during the early stages of disease. Ceftiofur is a bactericidal and broad

spectrum antibiotic representing the third-generation of cephalosporins. Many Gram-positive, Gram-negative and anaerobic bacteria of veterinary importance are susceptible to ceftiofur (Collard *et al.*, 2011). It is formulated as ceftiofur sodium, ceftiofur hydrochloride and ceftiofur crystalline-free acid suspension. Ceftiofur hydrochloride is mainly used to treat bovine respiratory disease associated with *P. multocida*, *Haemophilus somnus* and *Mannheimia haemolytica* (FDA, 1988). Besides, it is effective against acute foot rot and postpartum metritis in bovine (Papich, 2016). The antimicrobial sensitivity of *P. multocida* considerably varies and several strains have acquired resistance against certain frequently used antimicrobial agents (Baloch *et al.*, 2014). Accordingly, the periodic assessment of *in vitro* antimicrobial susceptibility becomes essential to forecast the clinical utility of antimicrobial drugs against *P. multocida* (Salmon *et al.*, 1996). The current project was designed to determine the *in vitro* efficacy of ceftiofur hydrochloride against bubaline *P. multocida*

following its preliminary identification on the basis of cultural, morphological and biochemical characteristics, and confirmation through polymerase chain reaction (PCR).

Materials and Methods

Experimental Drug and Bacterial Growth Media

The active pharmaceutical substance of ceftiofur hydrochloride was generously supplied by International Pharma Labs, Lahore, Pakistan. Blood agar (BA), brain heart infusion agar (BHIA), Mueller-Hinton broth (MHB), MacConkey's agar (MCA), brain heart infusion broth (BHIB) and urea agar base were procured from Himedia, Laboratories, Mumbai, India.

Test Organisms

The clinical isolate of *P. multocida* was collected by nasal swab sampling from a water buffalo (*Bubalus bubalis*) showing the characteristic manifestations of HS. Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan provided the reference isolate of *P. multocida* (serotype B:2, strain P₅₂).

Bacterial Cultivation, Purification and Characterization

The bacterial isolates were inoculated into BHIB, incubated at 37°C for 24 h and purified by subculturing on BA plates. Subsequently, the organisms were examined for failure of growth on MCA. Presumptive *P. multocida* isolates were characterized on the basis of Gram's staining, standard biochemical tests and fermentation reactions as described elsewhere (Songer and Post, 2005).

Pathogenicity Analysis

The pathogenicity of isolates was analyzed by challenging 6 weeks old Balb/C mice through intraperitoneal injections of 0.1 mL of bacterial culture containing 0.3x10⁸ colony forming units (CFU)/mL (Naz *et al.*, 2012). The organisms were re-isolated from dead mice, cultured on blood agar (BA) and confirmed by Gram's staining.

Molecular Identification

Pure broth cultures of both isolates were processed for *P. multocida* specie-specific PCR (PM-PCR) and HS-causing B serotype-specific PCR (HSB-PCR) using specie-specific and B serotype-specific primers, respectively (Table 1) as reported earlier (Townsend *et al.*, 1998). Bacterial DNA was isolated from purified cultures using the DNA extraction kit following the manufacturer's instructions (Vivantis technologies, Malaysia). Both of the aforementioned PCR assays were performed using the same reaction conditions. The reaction mixture (20 µL) consisted

of 10 µL of 2X Taq mastermix, 1 µL (10 pmoles) of each primer pair, 2 µL of template DNA and 6 µL of nuclease-free water. Initial denaturation, final denaturation and annealing were conducted at 95°C for 7 mins, 94°C for 1 min and 55°C for 1 min, respectively. The temperature was adjusted to 72°C for 1 min to carry out the initial extension. The terminal denaturation, annealing and extension steps were repeated for 30 times. The final extension was performed at 72°C for 7 mins. The PCR product (5 µL) and 100 bp DNA ladder were electrophoresed on 1.5% agarose gel, stained with 1% ethidium bromide, visualized and photographed by means of Omega Fluor™ Plus gel documentation system (Aplegen Gel Company, USA).

Broth Microdilution Assay for Estimation of MIC and MBC of Ceftiofur Hydrochloride

Preparation of stock solution: The stock solution was prepared by dissolving 100 mg of ceftiofur hydrochloride in 10 mL of distilled water to achieve a stock solution of 10 mg/mL. This stock solution was ten-fold diluted for three times to obtain a working solution of 10 µg/mL.

Standardization of bacterial inocula: Well-isolated, 3–5 bacterial colonies were inoculated into test tubes containing 5 mL of MHB and incubated at 37°C for 6 h. The final bacterial inocula containing 5x10⁵ CFU/mL were prepared by means of turbidity adjustment method.

Preparation of microdilution tray: The MIC and MBC of ceftiofur hydrochloride were determined against *P. multocida* using broth microdilution assay as per the guidelines of Clinical and Laboratory Standards Institute (C.L.S.I., 2013a). The flat-bottomed, 96-well plate was charged with 100 µL each of sequentially varying concentrations of ceftiofur hydrochloride (10–0.02 µg/mL), MHB and standardized culture to achieve the final volume of 300 µL per well. Whereas, growth control and sterility control wells contained 300 µL of bacterial inocula and MHB, respectively. The plate was incubated at 37°C and analyzed after 24 h.

Interpretation of end points: The minimum concentration of ceftiofur hydrochloride preventing the visible bacterial growth was referred to as MIC. Subsequently, 100 µL of suspension was collected from each well demonstrating the lack of bacterial growth (indicating the ceftiofur hydrochloride concentrations ≥ MIC) and subcultured on sterile BHIA plates. The plates were checked after being incubated at 37°C for 24 h and MBC was recorded as the lowest concentration of ceftiofur hydrochloride that reduced the original inoculum by 99.9% (Fernandez-Varon *et al.*, 2016).

In vitro Time-kill Kinetics

The *in vitro* time-kill kinetics of ceftiofur hydrochloride was calculated against *P. multocida* as described elsewhere (Fernandez-Varon *et al.*, 2016). Briefly, 10 µL of each

Table 1: Primers used for PCR-based identification of *P. multocida*

Specificity	Primer	Sequence (5'-3')	Amplicon size	Reference
<i>P. multocida</i>	KMT1T7	ATCCGCTATTACCCAGTGG	460 bp	Townsend <i>et al.</i> (1998)
	KMT1SP6	GCTGTAAACGAACTCGCCAC		
HS-causing B serotype	KTT72	AGGCTCGTTTGGATTATGAAG	620 bp	
	KTSP61	ATCCGCTAACACACTCTC		

ceftiofur hydrochloride concentration (0, 0.5, 1, 2, 4, 8 and 16 multiples of MIC) was added into 480 μ L of sterile MHB in individual test tubes. The samples were inoculated with 10 μ L of inocula in stationary phase to obtain a final bacterial density of $\sim 1 \times 10^8$ CFU/mL and incubated at 37°C. Viable counts were calculated at 0, 1, 2, 4, 6, 8, 12 and 24 h of incubation, and the results were expressed as CFU/mL.

Statistical Analysis

The *in vitro* time-kill kinetic data were analyzed by repeated measures analysis of variance (ANOVA) using the Statistical Package for Social Sciences (S.P.S.S. Inc., Chicago, I.L., U.S.A.), version 21.0. Bonferroni test was applied for multiple comparisons among the individual time points and ceftiofur concentrations. Differences were considered significant at *P*-value < 0.05.

Results

Phenotypic and Molecular Characteristics of *P. multocida*

The cultural, morphological and biochemical features of both isolates were identical to those typical for *P. multocida*. Luxuriant growth was observed on BA plates characterized by small, translucent, grayish, dew-drop like and non-hemolytic colonies following the overnight incubation at 37°C. BHIA plates inoculated with *P. multocida* also showed copious growth in terms of whitish gray, opaque colonies. Nevertheless, the isolates failed to grow on MCA. Examination of Gram's stained smear revealed bipolar coccobacilli. The isolates were positive for the production of catalase, oxidase and indole. While, negative results were documented for methyl red test, urease production and citrate utilization. Mice challenged with clinical isolate and reference strain died within 24 h after inoculation. *P. multocida* was successfully re-isolated from the heart blood of dead mice and confirmed through Gram's stained impression smear. Both isolates were validated as *P. multocida* type B on the basis of PM-PCR and HSB-PCR assays leading to amplification products of 460 bp and 620 bp, respectively (Fig. 1).

MIC and MBC of Ceftiofur Hydrochloride

The MIC and MBC of ceftiofur hydrochloride were 0.15 μ g/mL and 0.30 μ g/mL, respectively against the reference isolate. Nevertheless, slightly higher MIC and MBC values

of 0.30 μ g/mL and 0.62 μ g/mL demonstrated the relatively lower sensitivity of clinical isolate to ceftiofur hydrochloride. Overall, ceftiofur hydrochloride exerted adequate *in vitro* efficacy against *P. multocida* with estimated mean values of MIC and MBC as 0.22 μ g/mL and 0.45 μ g/mL, respectively.

In vitro Time-kill Kinetics

The *in vitro* time-kill curves of ceftiofur achieved over a period of 24 h against *P. multocida* have been presented in Fig. 2. Ceftiofur hydrochloride concentration below the MIC (0.5 x MIC) had no appreciable impact on bacterial growth. The bacteriostatic effect of ceftiofur hydrochloride was observed in terms of 1 \log_{10} bacterial reduction at concentrations equal to MIC (1 x MIC) and twice of MIC (2 x MIC) within 12 and 2 h of incubation, respectively. Ceftiofur hydrochloride concentrations of 2 x MIC, 4 x MIC, 8 x MIC and 16 x MIC exerted bactericidal effect against the reference isolate during 12, 6, 4 and 2 h, respectively. However, the colony counts of clinical isolate experienced a 3 \log_{10} reduction at concentrations of 4 x MIC, 8 x MIC and 16 x MIC after 8, 6 and 4 h, respectively (Fig. 2). Furthermore, the bacterial density was diminished by a factor of 4 \log_{10} at 4 x MIC in 12 h, 8 x MIC in 8 h and 16 x MIC in 6 h (Fig. 2). Bacterial population exposed to very high drug concentrations of 8 x MIC and 16 x MIC suffered from a 5 \log_{10} reduction during 12 h.

Overall, the bacterial killing pattern considerably varied with time and ceftiofur concentration. Multiple comparison analysis revealed consistent bacterial count during 0–6 h, followed by cumulative bacterial killing at time points of 8, 12 and 24 h (Fig. 3). Comparable cell counts were recorded for control and bacterial sample exposed to sub-MIC concentration of ceftiofur hydrochloride. Regardless of a distinct correlation between the MIC and bacterial killing, concentrations \geq MIC considerably reduced the bacterial density (Fig. 4).

Discussion

Prompt and effective treatment of a bacterial infection necessitates the current antimicrobial sensitivity data of causative agent (Wu *et al.*, 2018). This study aimed to determine the *in vitro* pharmacodynamics of ceftiofur hydrochloride against *P. multocida* isolated from water buffalo, following its identification on the basis of cultural, morphological and biochemical characteristics, and

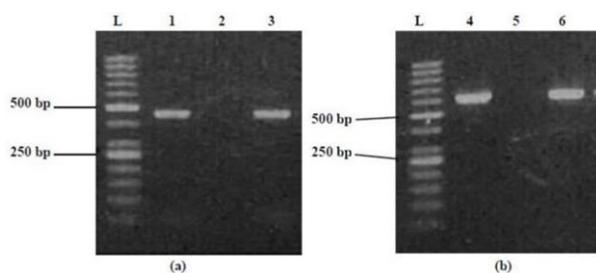


Fig. 1: PCR amplification products for molecular identification of *P. multocida*. (a); *P. multocida* specific PCR (PM-PCR) showing an amplification product of 460 bp and (b); HS causing B serotype specific PCR (HSB-PCR) indicating the amplification product of 620 bp. L; Ladder, 1 & 4; Reference isolate, 2 & 5; Negative control, 3 & 6; Clinical isolate

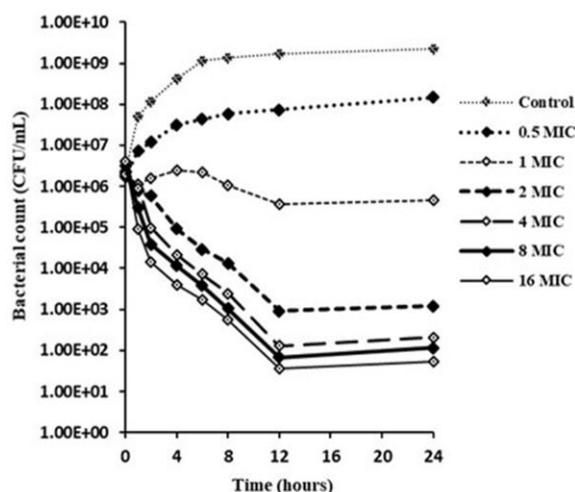


Fig. 2: Mean *in vitro* time-kill kinetics of ceftiofur hydrochloride against *P. multocida*

confirmation through PCR. The reported phenotypic attributes of tested isolates are congruent with the results of prior studies (Jabeen *et al.*, 2013; Mehmood *et al.*, 2016). Additionally, comparable pattern of pathogenicity has been previously demonstrated in mice (Kumar *et al.*, 2004; Naz *et al.*, 2012) and rabbits (Ahmad *et al.*, 2011) inoculated with *P. multocida*. The application of PCR assay has been recommended for rapid and efficient confirmation of *P. multocida* following the initial identification by culture characteristics and biochemical reactions (Verma *et al.*, 2015). Consistent with prior studies (Markam *et al.*, 2009; Dey *et al.*, 2014), the isolates were confirmed as *P. multocida* type B using PCR assays.

In accordance with previous reports, the reference isolate exhibited adequate susceptibility to ceftiofur hydrochloride (Jamali *et al.*, 2014; Cucco *et al.*, 2017), while, comparatively higher MIC and MBC values were recorded against the clinical isolate (Waraich *et al.*, 2017; Sidhu *et al.*, 2018). Moreover, preceding studies have indicated variable *in vitro* efficacy of ceftiofur against *P.*

multocida isolates recovered from different animal species. The MIC of ceftiofur was documented as 0.125 $\mu\text{g/mL}$ against *P. multocida* isolated from cattle (Post *et al.*, 1991; Katsuda *et al.*, 2013) and water buffalo (Nie *et al.*, 2016). Conversely, the MIC values of ceftiofur were $<0.25 \mu\text{g/mL}$ and $\leq 0.5 \mu\text{g/mL}$ against *P. multocida* isolates originated from goats (Clothier *et al.*, 2012) and pigs (Nedbalcová and Kučerová, 2013), respectively. More recently, the MIC and MBC of ceftiofur were documented as 0.15 $\mu\text{g/mL}$ and 0.28–0.32 $\mu\text{g/mL}$, respectively against *P. multocida* of caprine origin (Waraich *et al.*, 2017; Sidhu *et al.*, 2018). The relatively higher values of MIC and MBC recorded in current study could be attributed to potentially different bacterial sensitivity on account of specie-specific and geographical variations. Besides, the phenomenon of cross-resistance among β -lactam antibiotics and off-label use of ceftiofur have also been postulated to alter the bacterial sensitivity pattern (Persoons *et al.*, 2011; Berendsen *et al.*, 2012). Nevertheless, the currently observed MIC values of ceftiofur hydrochloride are well below the susceptibility breakpoint of $\leq 2 \mu\text{g/mL}$ for *P. multocida* as recommended by the Clinical and Laboratory Standards Institute (C.L.S.I., 2013b). Therefore, ceftiofur hydrochloride exerted adequate *in vitro* efficacy against *P. multocida* with estimated mean values of MIC and MBC as 0.22 $\mu\text{g/mL}$ and 0.45 $\mu\text{g/mL}$, respectively.

Time-kill assays efficiently determine the magnitude of bacterial killing at tested drug concentrations during a short period of time (Dorfman *et al.*, 2008). Bactericidal drugs typically provoke 99.9% (3 \log_{10}) reduction of the bacterial colony count (Dorfman *et al.*, 2008). Ceftiofur hydrochloride demonstrated bactericidal effect against the reference and clinical isolates at concentrations of 2 x MIC and 4 x MIC after 12 and 8 h, respectively. Previously, ceftiofur concentration 4 times higher than MIC has been associated with 1 \log_{10} and 4 \log_{10} diminution of *P. multocida* exposed for 8 and 24 h, respectively (Waraich *et al.*, 2017). Contrary to our findings, ceftiofur concentration two times greater than MIC resulted in bactericidal activity against *Mannheimia haemolytica*, following an exposure time of 24 h (Fernandez-Varon *et al.*, 2016). Compliant with earlier reports, the presented data on time-kill kinetics validate the time-dependent bactericidal activity of ceftiofur hydrochloride against *P. multocida* (Craig, 2003; Waraich *et al.*, 2017). Conversely, very high concentrations of ceftiofur (20–30x MIC) resulted in concentration-dependent bactericidal activity (Waraich *et al.*, 2017).

Conclusion

The results of current study demonstrated appropriate *in vitro* efficacy and time-dependent bactericidal activity of ceftiofur hydrochloride against *P. multocida* isolated from water buffalo. Therefore, the rational use of β -lactam antibiotics is requisite to preclude the likelihood of cross-resistance and preserve the antibacterial activity of ceftiofur.

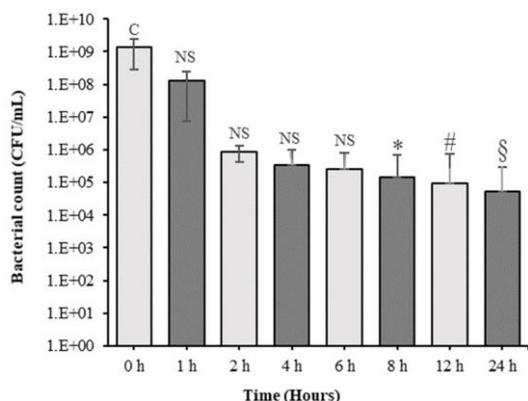


Fig. 3: Effect of time on kill kinetics of *P. multocida*. C; control, NS; non-significant (P -value > 0.05), *; P -value < 0.05, #; P -value < 0.02, §; P -value < 0.01 (in comparison with control group)

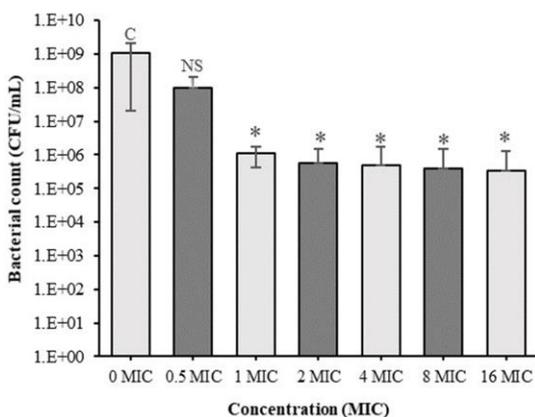


Fig. 4: Effect of ceftiofur hydrochloride concentration on kill kinetics of *P. multocida*. C; control, NS; non-significant (P -value > 0.05), *; P -value < 0.05 (in comparison with control group), bars showing similar asterisks indicate the lack of significant difference among corresponding groups

Additionally, controlled clinical trials are recommended for evaluating the therapeutic effectiveness of ceftiofur hydrochloride in target animal species.

Acknowledgement

The authors acknowledge M/S International Pharma Labs, Lahore, Pakistan for the provision of ceftiofur hydrochloride reference standard. The authors are also thankful to Microbiology Section, Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan for providing the reference isolate of *P. multocida*.

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[Received 26 Feb 2019; Accepted 22 May 2019; Published (online) 10 Nov 2019]