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Hematological and antimicrobial resistance patterns in buffalo calves with *Hemorrhagic septicemia*: A clinical study

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Abstract

Hemorrhagic septicemia (HS) is a life-threatening disease caused by *Pasteurella multocida*, primarily affecting buffaloes. This study was conducted on a total of 46 buffaloes, including 23 clinically affected animals showing symptoms of *Hemorrhagic septicemia* (HS) characterized by sudden onset, high rise in body temperature, anorexia, depression, oedematous swelling on throat, brisket and upper dewlap region, dyspnoea, nasal discharge, salivation and reluctance to move that were presented at the Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya (Uttar Pradesh), India. Additionally, 23 apparently healthy buffaloes maintained at the Department of Livestock Farm Complex, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya (Uttar Pradesh), India, were included as controls. Diagnosis was established through blood smear examination and bacterial culture using Giemsa and Gram staining techniques, respectively, along with biochemical characterization by observing hemolysis on blood agar. The causative organism, *Pasteurella multocida*, demonstrated characteristic bipolar staining in both blood and Gram-stained smears. Complete blood count (CBC) and antibiotic susceptibility testing (ABST) were also performed. Hematological findings revealed deviations consistent with infection, and ABST results showed notable antibiotic resistance. High sensitivity was noted for ceftriaxone-sulbactam (82.6%), vancomycin (78.30%), ofloxacin (78.3%), ciprofloxacin (73.91%), amikacin (69.57%), gentamicin (65.22%), enrofloxacin (65.22%) and chloramphenicol (60.9%).

The study aimed to evaluate alterations in hematological parameters and to assess the antibiotic resistance patterns of the isolated organisms.

Keywords: *Hemorrhagic septicemia*, *Pasteurella multocida*, buffalo, antimicrobial resistance Blood smear diagnosis

Introduction

The pathogenic bacterium *Pasteurella multocida* (*P. multocida*) is a major concern in veterinary medicine because it can cause a variety of diseases in livestock, such as respiratory infections, pneumonia, and *Hemorrhagic septicemia* (Harper *et al.*, 2006; Hill and Brown *et al.*, 2011; Souza, 2009) [6, 7, 16]. This bacteria causes significant financial losses in the agricultural sector in India and is common in a variety of animal species, such as pigs, sheep, cattle, and buffalo (Govindaraj *et al.*, 2017) [5]. Additionally, *P. multocida*'s zoonotic potential poses a threat to public health, especially in areas where intimate human-animal interactions are common (Wilson and Ho, 2013) [19].

Antibiotic-resistant *P. multocida* strains are becoming more common, which is a serious problem since they make treating infections more difficult and raise the possibility that resistant strains could spread among animal populations (Oh *et al.*, 2018) [10]. There have been reports of resistance to popular antibiotics including ampicillin, tetracycline, and streptomycin in different parts of India, which calls for a more thorough investigation of the underlying resistance mechanisms (Uddin *et al.*, 2021) [17].

The existence of integrative and conjugative elements (ICEs) and mobile genetic elements, which promote the horizontal transfer of resistance genes between bacteria, is frequently the cause of antibiotic resistance (Partridge *et al.*, 2018) ^[13]. These genetic elements support the persistence and dissemination of antibiotic resistance in *P. multocida*, in conjunction with efflux pumps and other resistance mechanisms (Li *et al.*, 2015) ^[9]. Knowing these processes is crucial for controlling existing infections as well as anticipating and stopping resistant strain outbreaks in the future.

Whole-genome sequencing and molecular methods have emerged as crucial instruments for detecting *P. multocida* and describing its resistance patterns in order to solve this problem. These techniques enable the identification of integrative and conjugative elements (ICEs), mobile genetic elements, and resistance genes—all of which are crucial for the spread of resistance (Peng *et al.*, 2019) ^[14]. These kinds of insights are essential for creating focused interventions to control and lessen the effects of *P. multocida* strains that are resistant to antibiotics. This study's main goal is to look at the genetic processes that underlie antibiotic resistance in *P. multocida* strains that have been isolated from different animal hosts in India. In order to better understand *P. multocida* resistance patterns and develop management and control measures, this study will analyze resistance genes, mobile genetic elements, and other genomic aspects.

Materials and Methods

The study was conducted on a total of 46 buffaloes, including 23 clinically affected animals showing symptoms of *hemorrhagic septicemia* (HS) that were presented at the Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya (Uttar Pradesh), India. Additionally, 23 apparently healthy buffaloes maintained at the Department of Livestock Farm Complex, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya (Uttar Pradesh), India, were included as controls. The study aimed to evaluate alterations in hematological parameters and to assess the antibiotic resistance patterns of the isolated organisms.

Blood Sample Collection

Blood Samples for Hematology

5 ml of blood was collected aseptically from the jugular vein using a sterile 18-gauge needle and transferred into an EDTA vacutainer for Complete Blood Count (CBC) analysis using an automated hematology analyzer.

Blood Samples for Bacterial Isolation

An additional 5 ml of blood was collected in a plain sterile vacutainer (without anticoagulant). Immediately after collection and before clotting, 1 ml of whole blood was aseptically transferred into 9 ml of Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 hours to promote bacterial growth.

Nasal Swab Collection

Using a sterile cotton swab, a nasal sample was gently taken from the same animal. The swab was inserted into the nasal cavity, rotated, and placed into transport media.

Bacterial Isolation and Identification

- After enrichment in BHI broth, a loopful of the culture

was streaked on 5% sheep blood agar and incubated at 37°C for 24–48 hours under aerobic conditions.

- **Colonial Morphology:** Typical colonies of *Pasteurella multocida* were identified based on their small, grayish, non-hemolytic, mucoid appearance.
- **Gram's Staining:** Smears were prepared from colonies and stained by Gram's method. *P. multocida* appeared as Gram-negative coccobacilli showing characteristic bipolar ("safety-pin") appearance.
- **Catalase Test:** Performed by placing a drop of 3% hydrogen peroxide on a clean glass slide and adding a bacterial colony. Immediate bubbling indicated a positive catalase reaction.
- **Hemolysis on Blood Agar:** The presence or absence of hemolysis was noted. *P. multocida* typically exhibits non-hemolytic behavior, although variations may occur.

Complete Blood Count (CBC)

Hematological analysis was done using an automated cell counter (e.g., Nihon Koden). The following parameters was evaluated:

- Hemoglobin (Hb)
- Packed Cell Volume (PCV)
- Total Erythrocyte Count (TEC)
- Total Leukocyte Count (TLC)
- Differential Leukocyte Count (DLC)
- Platelet count
- Mean Corpuscular Volume (MCV)
- Mean Corpuscular Hemoglobin (MCH)
- Mean Corpuscular Hemoglobin Concentration (MCHC)

Antibiotic Sensitivity Testing

- Antibiotic susceptibility of the isolated strains was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. A bacterial suspension equivalent to 0.5 McFarland standards was prepared and spread evenly on the agar surface. Commercial antibiotic disks were placed, and plates were incubated at 37°C for 18–24 hours. Zones of inhibition were measured and interpreted as per CLSI guidelines. The following antibiotic discs were used

S. No	Antibiotic disc	Symbol	Conc. (mcg)
1.	Ceftriaxone sulbactam	(CIS)	30/15
2.	Cefoxitin	(Cx)	30
3.	Ciprofloxacin	(CIP)	5
4.	Ofloxacin	(OF)	5
5.	Cotrimoxazole/ Sulpha Trimethoprim	(COT)	25
6.	Vancomycin	(VA)	30
7.	Methicillin	(MET)	10
8.	Tetracycline	(TE)	30
9.	Azithromycin	(AZM)	30
10.	Gentamicin	(GEN)	10
11.	Amikacin	(AK)	30
12.	Penicillin	(P)	10 units
13.	Enrofloxacin	(EX)	10
14.	Chloramphenicol	(C)	50
15.	Norfloxacin	(NX)	10
16.	Oxacillin	(OX)	5

Results and Discussion

Giemsa-stained blood smears revealed the presence of bipolar organisms consistent with *Pasteurella multocida*. Hematological analysis of 23 buffalo calves demonstrated significant deviations from standard reference ranges,

indicative of active infection. The TEC (8.3 ± 0.2 million/cu mm) and Hb (12.1 ± 0.4 gm/dl) were within upper normal limits, suggesting uncompromised oxygen-carrying capacity. Elevated TLC ($16,000 \pm 500$ /cu mm) and marked neutrophilia ($72.9 \pm 3.5\%$) reflected acute inflammation, while lymphopenia ($17.9 \pm 2\%$) suggested immune suppression. Monocytes ($0.1 \pm 0.1\%$) were low, possibly due to tissue sequestration. Eosinophils ($9.1 \pm 0.8\%$) remained within normal limits but could suggest parasitic co-infections. RBC indices including MCV (46.7 ± 0.9 fl), MCH (14.6 ± 0.2 pg), and MCHC (31.3 ± 0.3 gm/dl) remained within expected ranges, indicating no macrocytic or microcytic changes. Platelet count ($245,000 \pm 12,000$ /cu mm) was also within normal limits, suggesting no coagulation abnormalities.

Arif Zafar *et al.*, (2009) ^[1], also reported concentration of haemoglobin in HS suffering animals was lower than the reference (reference value: 12-15 g/ dl) values. Haematocrit was also decreased than the normal values (reference value: 30-35%) in diseased buffaloes. Cultural characteristics on nutrient agar and blood agar confirmed *P. multocida*, with Gram staining revealing gram-negative rods with bipolar morphology. Colonies exhibited the typical greyish-white appearance with "mousy" odor.

After incubation for 24 h at 37 °C, surface colonies on sheep blood agar plates were smooth, spherical, shiny, grayish or yellowish, nonhemolytic, and 1-2 mm in diameter (Piorunek *et al.*, 2023) ^[15]. The type of capsule and level of encapsulation determine colony size. In rich broth, good growth was obtained; on MacConkey agar, no growth was

observed (Desem *et al.*, 2023) ^[4]. The organism exhibits bipolar staining with Leishman, methylene blue, or Giemsa staining in fresh cultures, blood smears, exudates, and tissues of infected animals (Panna *et al.*, 2015) ^[12].

Antimicrobial susceptibility testing of isolates from nasal swabs indicated high resistance to methicillin (95.65%), cefoxitin (82.60%), oxacillin (82.6%) and penicillin (78.3%). Moderate resistance was found for Cotrimoxazole/ Sulpha Trimethoprim (65.22%), norfloxacin (65.22%), Chloramphenicol (56.52%), tetracycline (43.5%), azithromycin (30.43%), and gentamicin (34.78%). High sensitivity was noted for ceftriaxone-sulbactam (82.6%), vancomycin (78.30%), ofloxacin (78.3%), ciprofloxacin (73.91%), amikacin (69.57%), gentamicin (65.22%), enrofloxacin (65.22%) and chloramphenicol (60.9%).

These findings are consistent with previous reports (Borah *et al.*, 2014; Kumar *et al.*, 2020) ^[3, 8], confirming neutrophilia as a key inflammatory marker in HS. The lymphopenia and monocytopenia reflect acute-phase immune alterations. The antibiotic resistance trends echo global concerns regarding multidrug-resistant strains of *Pasteurella multocida* (Watts and Sweeney, 2017) ^[18].

Though azithromycin showed moderate resistance (39.1%) in this study, some reports indicate its *in vivo* effectiveness. Continued surveillance and tailored antibiotic regimens are necessary. The Kirby-Bauer method remains a reliable tool for guiding treatment decisions and addressing antibiotic resistance in clinical settings (Bauer *et al.*, 1966; O'Neill, 2016) ^[2, 11].

Table 1: Hematological Parameters in Buffalo Calves Infected with *Hemorrhagic Septicemia*

Parameters	HS Animals (Mean $\pm$ SE)	95% CI (HS)	Healthy Animals (Mean $\pm$ SE)	95% CI (Healthy)	P-value	Interpretation
Total erythrocyte count	8.3 ± 0.2 million/cu mm	7.91 – 8.69	7.5 ± 0.3 million/cu mm	6.91 – 8.09	0.0012	Significant
Total leukocyte count (TLC)	16000 ± 500 /cu mm	15020 – 16980	11000 ± 400 /cu mm	10216 – 11784	< 0.0001	Highly Significant
Hemoglobin (Hb)	12.1 ± 0.4 gm/dl	11.32 – 12.88	10.5 ± 0.3 gm/dl	9.91 – 11.09	< 0.0001	Highly Significant
Packed cell volume	$38.6 \pm 1.1\%$	36.44 – 40.76	$36.0 \pm 1.0\%$	34.04 – 37.96	0.0075	Significant
Neutrophils (N)	$72.9 \pm 3.5\%$	66.02 – 79.78	$35.0 \pm 2.0\%$	31.08 – 38.92	< 0.0001	Highly Significant
Lymphocyte (L)	$17.9 \pm 2.0\%$	14.02 – 21.78	$55.0 \pm 3.0\%$	49.12 – 60.88	< 0.0001	Highly Significant
Monocyte (M)	$0.1 \pm 0.1\%$	-0.096 – 0.296	$5.0 \pm 1.0\%$	3.04 – 6.96	< 0.0001	Highly Significant
Eosinophil (E)	$9.1 \pm 0.8\%$	7.54 – 10.66	$6.0 \pm 0.5\%$	5.02 – 6.98	< 0.0001	Highly Significant
Mean Corpuscular Volume (MCV)	46.7 ± 0.4 fl	45.92 – 47.48	50.0 ± 0.5 fl	49.02 – 50.98	0.0003	Significant
Mean Corpuscular Hemoglobin (MCH)	14.6 ± 0.2 pg	14.21 – 14.99	15.5 ± 0.3 pg	14.91 – 16.09	0.0031	Significant
Mean Corpuscular Hemoglobin Concentration (MCHC)	31.3 ± 0.1 gm/dl	31.10 – 31.50	32.0 ± 0.4 gm/dl	31.22 – 32.78	0.0005	Significant
Platelet Count	$245,000 \pm 10,000$ /cu mm	225,400 – 264,600	$310,000 \pm 12,000$ /cu mm	286,480 – 333,520	< 0.0001	Highly Significant

Table 2: Antibiotic Resistance Profile of *Pasteurella multocida* Isolated from Buffalo Calves (no. of nasal swab sample- 23)

S. No	Antibiotic disc	Resistant zone	Sensitive zone
1.	Ceftriaxone sulbactam (CIS)	4 (17.4)	19 (82.6%)
2.	Cefoxitin (Cx)	19 (82.6%)	4 (17.4)
3.	Ciprofloxacin (CIP)	6 (26.09%)	17 (73.91)
4.	Ofloxacin (OF)	5 (21.7%)	18 (78.3)
5.	Cotrimoxazole/ Sulpha Trimethoprim (COT)	15 (65.22%)	8 (34.78%)
6.	Vancomycin (VA)	5 (21.7%)	18 (78.3)
7.	Methicillin (MET)	22 (95.65%)	1 (4.35%)
8.	Tetracycline (TE)	10 (43.48%)	13 (56.52%)
9.	Azithromycin (AZM)	7 (30.43%)	16 (69.57%)
10.	Gentamicin (GEN)	8 (34.78%)	15 (65.22%)
11.	Amikacin (AK)	7 (30.43%)	16 (69.57%)
12.	Penicillin (P)	18 (78.3)	5 (21.7%)
13.	Enrofloxacin (EX)	8 (34.78%)	15 (65.22%)
14.	Chloramphenicol (C)	13 (56.52%)	10 (43.48%)
15.	Norfloxacin (NX)	15 (65.22%)	8 (34.78%)
16.	Oxacillin (OX)	19 (82.6%)	4 (17.4)



Fig 1: Bipolar Staining of *Pasteurella multocida* in Blood Smear



Fig 2: Bacterial Growth of *Pasteurella multocida* on Nutrient Agar

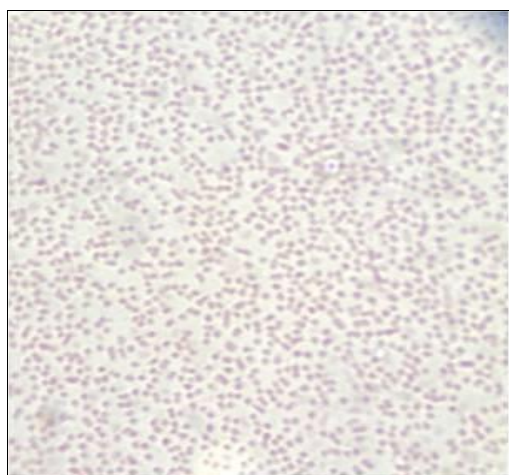


Fig 3: Gram Staining Showing Pink Bipolar Rods of *Pasteurella multocida*



Fig 4: Hemolytic Growth of *Pasteurella multocida* on Blood Agar

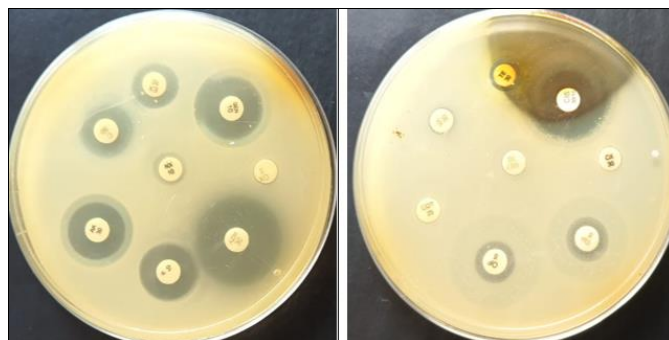
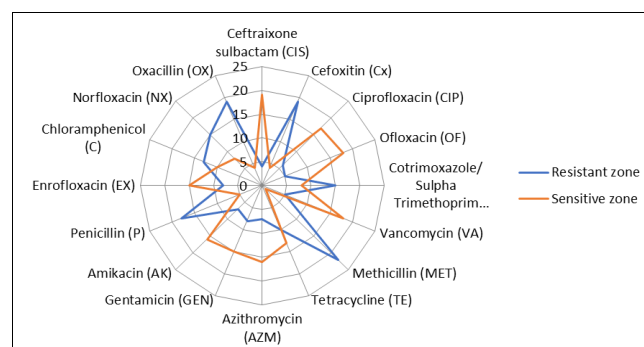


Fig 5: Antibiotic Susceptibility Test for *Pasteurella multocida* Isolated from Nasal Swab



Conclusion

In summary, the hematological findings in this group of buffalo calves strongly support the presence of an acute bacterial infection, aligning with the clinical presentation of Haemorrhagic Septicemia. Marked neutrophilia, lymphopenia, and elevated total leukocyte counts were consistent with the systemic inflammatory response observed in septicemic conditions caused by *Pasteurella multocida*. Early detection through hematological profiling, as demonstrated in this study, is crucial for timely intervention and reducing HS-associated mortality. Furthermore, antimicrobial susceptibility testing revealed substantial resistance to commonly used antibiotics such as methicillin, penicillin, and oxacillin. However, promising efficacy was observed with ceftriaxone-sulbactam, ciprofloxacin, amikacin, gentamicin and azithromycin indicating their potential utility in therapeutic management.

Conflict of Interest

Not available

Financial Support

Not available

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