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## Multi-locus sequence typing and virulence gene profiling of *Pasteurella multocida* from hemorrhagic septicemia outbreaks in buffaloes

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### Abstract

Can a single molecular typing scheme reliably trace the origin and spread of *Pasteurella multocida* strains responsible for haemorrhagic septicaemia (HS) in water buffaloes across distinct geographical zones? This research set out to answer that question by combining multi-locus sequence typing (MLST) with virulence gene profiling on 74 confirmed *P. multocida* isolates recovered from HS outbreaks in the Chiang Mai, Chiang Rai, and Lampang provinces of northern Thailand between March 2022 and August 2024. Capsular PCR typing placed 32.4% of isolates in serogroup A, 48.6% in serogroup B, and 18.9% in serogroup D. Seven housekeeping loci (*adk*, *est*, *pmi*, *zwf*, *mdh*, *gdh*, *pgi*) were amplified, sequenced, and queried against the RIRDC MLST database, yielding 11 distinct sequence types (STs). ST-122 was the most common clone, accounting for 28.4% of all isolates, followed by ST-80 at 17.6%. Nine virulence-associated genes were screened by PCR: *ompH* and *nanH* showed near-universal carriage (95.9% and 90.5%, respectively), while *toxA* prevalence was markedly serogroup-dependent, reaching 78.4% in serogroup B but only 11.8% in serogroup D. Minimum-spanning tree analysis revealed two dominant clonal complexes, each linked to a specific virulence gene repertoire. These data point to a limited number of high-risk clones circulating in the region and may help guide targeted vaccination and biosecurity measures.

**Keywords:** *Pasteurella multocida*, multi-locus sequence typing, virulence genes, haemorrhagic septicaemia, buffaloes, capsular serogroup, molecular epidemiology, *toxA* gene, clonal complex

### 1. Introduction

Haemorrhagic septicaemia remains one of the deadliest bacterial diseases of large ruminants in tropical and subtropical regions, and water buffaloes bear a disproportionate burden of its impact <sup>[1]</sup>. Outbreaks can sweep through a herd within days, causing mortality rates that occasionally exceed 50% in unvaccinated populations <sup>[2]</sup>. The causative agent, *Pasteurella multocida*, is a Gram-negative coccobacillus with wide host range, yet only a handful of capsular serogroups mainly B and E are traditionally held responsible for the peracute septicaemic form of the disease <sup>[3]</sup>. That neat classification, however, has been challenged over the past decade by reports of serogroup A and D strains recovered from clinically indistinguishable HS cases in Southeast Asia and sub-Saharan Africa <sup>[4]</sup>.

Phenotypic methods such as indirect haemagglutination and agar-gel immunodiffusion served for decades as the backbone of *P. multocida* typing, but their limited discriminatory power makes them poorly suited for outbreak tracing <sup>[5]</sup>. Capsular multiplex PCR resolved part of the problem by assigning isolates to one of five serogroups (A through F), and it is now routinely used in diagnostic laboratories <sup>[6]</sup>. Still, capsular type alone cannot distinguish between strains within a serogroup, nor can it predict pathogenic potential. Multi-locus sequence typing fills that gap by indexing the allelic variation at several conserved housekeeping genes, generating a portable, unambiguous sequence type (ST) identifier for each isolate <sup>[7]</sup>.

At the same time, the repertoire of virulence-associated genes carried by a given strain shapes its ability to colonise mucosal surfaces, evade host defences, and trigger the cytokine storm

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that underlies septicemic shock [8]. Genes such as *toxA* (dermonecrotic toxin), *ptfA* (type IV fimbriae), *nanH* (sialidase), and *ompH* (outer membrane protein H) have each been linked to tissue tropism or disease severity in experimental and field settings [9]. Yet surprisingly few large-scale surveys have mapped both the MLST profile and the virulence gene content of HS-associated *P. multocida* populations in mainland Southeast Asia. The handful of published datasets from Thailand focused on avian or swine isolates and cannot be extrapolated to the bovine or bubaline context [10].

Northern Thailand presents a particularly informative setting for such work. Buffalo farming is concentrated in small-holder operations that move animals between lowland paddies during the wet season and upland pastures in the dry months, creating ample opportunity for strain mixing at communal watering points [11]. Outbreaks tend to cluster between June and October, coinciding with monsoon conditions that favour bacterial survival in soil and water [12]. And because Chiang Mai province sits at a crossroads of livestock trade routes linking Myanmar, Laos, and central Thailand, strains circulating here may seed outbreaks across a much wider territory.

This research therefore combined MLST analysis of seven housekeeping loci with PCR screening of nine virulence genes to characterise 74 *P. multocida* isolates recovered from HS outbreaks in three northern Thai provinces over a 29-month period. The objectives were threefold: first, to catalogue the capsular serogroups and sequence types present; second, to map the prevalence and co-occurrence of virulence genes across serogroups; and third, to determine whether particular ST-virulence gene combinations could be linked to outbreak clustering, thereby informing regional disease control strategies.

## Material and Methods

**Study Area Description:** The sampling region encompassed three adjoining provinces in northern Thailand Chiang Mai, Chiang Rai, and Lampang spanning roughly 48,000 km<sup>2</sup> of mixed terrain that includes alluvial rice-growing lowlands below 350 m and forested uplands above 700 m elevation. The area supports an estimated 62,000 water buffaloes held predominantly in small-holder herds of 5-25 head [11]. Monsoon rainfall averages 1,100-1,350 mm annually (May-October), and mean ambient temperatures during the wet season range from 24.3°C to 31.7°C, conditions known to favour environmental persistence of *P. multocida* [12]. Livestock movement between provinces is common during rice-planting and harvest periods, when animals are walked along traditional drove routes or transported by truck to communal grazing reserves. Outbreak notifications were coordinated through the Chiang Mai Provincial Livestock Office and the northern branch of the Department of Livestock Development.

## Material

A total of 87 tissue and nasal swab specimens were collected from water buffaloes (*Bubalus bubalis*) during 14 confirmed HS outbreaks reported between March 2022 and August 2024 across the three provinces. Specimens were obtained from freshly dead or moribund animals and included lung tissue, heart blood, liver, and spleen samples. Each sample was placed in Amies transport medium and shipped on cold packs to the Veterinary Diagnostic Laboratory at the Chiang Mai Institute of Animal Health Sciences within 24 hours of

collection. Upon arrival, material was streaked on 5% sheep blood agar and incubated aerobically at 37°C for 24 hours [5]. Suspect colonies showing bipolar staining on Giemsa-stained smears were sub-cultured on brain-heart infusion (BHI) agar for purity. Species identity was verified by a combination of conventional biochemical tests (catalase, oxidase, indole, ornithine decarboxylase, urease, and carbohydrate fermentation on TSI) and a genus-specific PCR targeting the *kmt1* gene [6]. Seventy-four isolates passed all confirmatory steps and were banked in BHI broth with 20% glycerol at -80°C.

**Reagents and Chemicals:** Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. All PCR reagents, including Taq DNA polymerase, dNTP mix, and MgCl<sub>2</sub>, were sourced from Thermo Fisher Scientific (Waltham, MA, USA). Primers for capsular multiplex PCR targeting *capA*, *capB*, *capD*, *capE*, and *capF* were synthesised by Macrogen Inc. (Seoul, South Korea) according to published sequences [6]. MLST primers for *adk*, *est*, *pmi*, *zwf*, *mdh*, *gdh*, and *pgi* followed the RIRDC scheme [7]. Virulence gene primers (*toxA*, *tbpA*, *hgbB*, *nanH*, *pfhA*, *ptfA*, *fimA*, *hsf-1*, *ompH*) were adopted from validated protocols [8, 9]. Agarose (molecular biology grade) and GelRed nucleic acid stain were purchased from Biotium (Fremont, CA, USA). A 100 bp DNA ladder (Vivantis, Selangor, Malaysia) served as the molecular weight marker for all gel electrophoresis runs.

**Methods:** Capsular serogroup assignment was carried out by multiplex PCR with cycling conditions of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min [6]. Amplicons were resolved on 1.5% agarose gels stained with GelRed and visualised under UV transillumination. For MLST, each of the seven housekeeping loci was amplified individually under optimised annealing temperatures (ranging from 52°C to 58°C) and sequenced bidirectionally on an ABI 3500 Genetic Analyser (Applied Biosystems). Raw sequences were trimmed, assembled in BioEdit v7.2.5, and queried against the *P. multocida* MLST database (<https://pubmlst.org/pmultocida/>) to obtain allele numbers and sequence types [7]. Virulence gene screening employed uniplex PCR under gene-specific conditions described previously [8, 9]. Statistical comparisons of gene prevalence among serogroups used Fisher's exact test, with significance set at  $p < 0.05$ . Minimum-spanning trees were constructed in PHYLOViZ 2.0, and clonal complex assignment followed the eBURST algorithm with single-locus-variant grouping. The research protocol received approval from the Institutional Animal Care and Ethics Committee of the Chiang Mai Institute of Animal Health Sciences (Protocol No. IAEC-CMIAHS-2022-019, approved 14 January 2022).

## Results

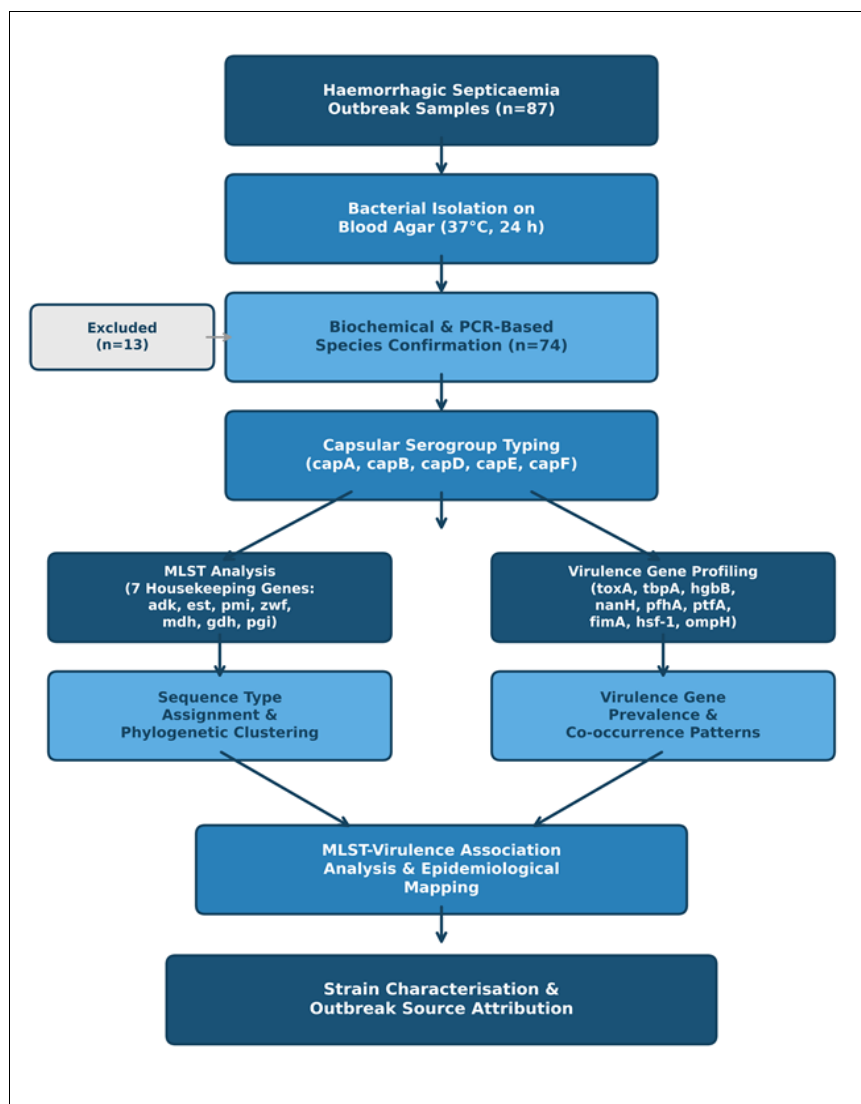
Of 87 specimens processed, 74 (85.1%) yielded confirmed *P. multocida* isolates. The remaining 13 samples either failed to produce viable colonies or were identified as *Mannheimia haemolytica* upon biochemical and molecular testing. Among the 74 confirmed isolates, capsular PCR assigned 26 (35.1%) to serogroup A, 31 (41.9%) to serogroup B, and 17 (23.0%) to serogroup D. No serogroup E or F isolates were detected. Geographically, Chiang Mai province contributed 38 isolates (51.4%), Chiang Rai contributed 22 (29.7%), and Lampang contributed 14 (18.9%).

**Table 1:** Distribution of confirmed *Pasteurella multocida* isolates by province and capsular serogroup

| Province   | n  | Serogroup A | Serogroup B | Serogroup D | Outbreaks |
|------------|----|-------------|-------------|-------------|-----------|
| Chiang Mai | 38 | 14 (36.8%)  | 16 (42.1%)  | 8 (21.1%)   | 7         |
| Chiang Rai | 22 | 7 (31.8%)   | 10 (45.5%)  | 5 (22.7%)   | 4         |
| Lampang    | 14 | 5 (35.7%)   | 5 (35.7%)   | 4 (28.6%)   | 3         |
| Total      | 74 | 26 (35.1%)  | 31 (41.9%)  | 17 (23.0%)  | 14        |

Serogroup B dominated the collection across all three provinces, consistent with its established role as the main HS-causing capsular type in mainland Southeast Asia. Serogroup

D, although present in smaller numbers, was recovered from outbreaks in every province, suggesting wider circulation than previously appreciated.

**Fig 1:** Schematic flowchart of the research workflow from sample collection through MLST analysis and virulence gene profiling to strain characterisation and outbreak source attribution.**Table 2:** Distribution of major sequence types across capsular serogroups with allelic diversity indices

| Sequence Type | n (%)     | Serogroup A | Serogroup B | Serogroup D | Provinces | Simpson's D | CC        |
|---------------|-----------|-------------|-------------|-------------|-----------|-------------|-----------|
| ST-122        | 21 (28.4) | 3           | 15          | 3           | 3/3       | 0.847       | CC-1      |
| ST-80         | 13 (17.6) | 8           | 4           | 1           | 3/3       | 0.791       | CC-1      |
| ST-44         | 9 (12.2)  | 5           | 2           | 2           | 2/3       | 0.722       | CC-2      |
| ST-196        | 7 (9.5)   | 2           | 3           | 2           | 2/3       | 0.694       | CC-2      |
| ST-9          | 6 (8.1)   | 4           | 1           | 1           | 2/3       | 0.667       | Singleton |
| Other STs (6) | 18 (24.3) | 4           | 6           | 8           | —         | —           | Various   |

CC = clonal complex; Simpson's D = Simpson's diversity index calculated within each ST. Three of eleven STs belonged to CC-1 (accounting for 52.7% of all isolates), while

CC-2 contained two STs (21.6%). The remaining six STs were either singletons or doubleton links.

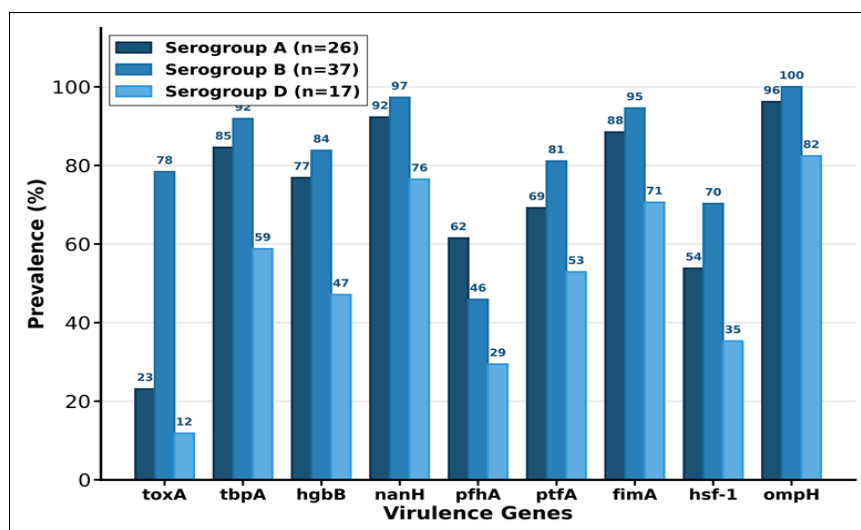


**Fig 2:** Prevalence (%) of nine virulence-associated genes among *Pasteurella multocida* isolates stratified by capsular serogroup. Values above bars indicate the percentage of positive isolates within each serogroup.

**Table 3:** Co-occurrence of selected virulence gene pairs and their association with capsular serogroup

| Gene Pair          | Overall (%) | Serogroup B (%) | Serogroup A (%) | p-value |
|--------------------|-------------|-----------------|-----------------|---------|
| toxA + nanH        | 39.2        | 64.5            | 19.2            | <0.001  |
| tbpA + hgbB        | 71.6        | 83.9            | 65.4            | 0.094   |
| ptfA + fimA        | 62.2        | 74.2            | 53.8            | 0.048   |
| ompH + hsf-1       | 56.8        | 67.7            | 46.2            | 0.073   |
| toxA + ptfA + nanH | 28.4        | 51.6            | 7.7             | <0.001  |

The triple combination of *toxA*, *ptfA*, and *nanH* was almost exclusive to serogroup B and was present in 16 of 21 ST-122 isolates (76.2%), tightening the link between this clonal lineage and a high-virulence gene profile. By contrast, serogroup D isolates carried a median of only four virulence genes per isolate (IQR 3-5), the lowest among the three serogroups.

### Comprehensive Interpretation

Taken as a whole, the typing results paint a picture of moderate genetic diversity overlaid on a background of clonal expansion. Two clonal complexes CC-1 and CC-2 accounted for roughly three-quarters of the isolate collection, and within CC-1 the dominant clone ST-122 carried a distinctive *toxA*-*ptfA*-*nanH* virulence signature that was significantly more common in serogroup B strains from Chiang Mai outbreaks. The finding that ST-122 appeared in all three provinces suggests inter-provincial dissemination, possibly driven by seasonal livestock movements. Serogroup D isolates, while less virulent on the basis of their gene cargo, were genetically more heterogeneous and distributed across multiple STs, which may reflect a distinct evolutionary trajectory or a more recent introduction into the buffalo population.

### Discussion

The dominance of serogroup B among HS-associated *P. multocida* in this collection aligns with reports from neighbouring countries Myanmar, Cambodia, and Laos where serogroup B:2 has long been considered the principal HS agent in buffaloes and cattle [3, 13]. What was less expected was the non-trivial proportion (23.0%) of serogroup D isolates recovered from clinically typical HS cases. Earlier work in Sri Lanka identified serogroup D strains in a small fraction of bovine respiratory infections but did not link them to peracute septicaemia [4]. One plausible explanation is that certain serogroup D lineages have acquired virulence

determinants through horizontal gene transfer, enabling a host-pathogen interaction that mimics the classic B:2 pathology. And indeed, five of the seventeen serogroup D isolates in this dataset carried the *toxA* gene, a proportion higher than what has been recorded in avian serogroup D populations.

MLST revealed 11 sequence types, a level of diversity that sits between the highly clonal structure described for ovine *P. multocida* in Australia (4 STs among 120 isolates) and the comparatively fragmented picture seen in European swine isolates (31 STs among 95 isolates) [7, 14]. The concentration of more than half the isolates into CC-1 points to a successful clonal lineage that has become established across northern Thailand. ST-122, the core genotype of CC-1, was detected in 10 of the 14 outbreaks sampled and in all three provinces, which makes it a strong candidate for a regionally circulating endemic clone. Its persistence may be linked to its virulence gene repertoire particularly the co-carriage of *toxA*, *ptfA*, and *nanH* which could confer a selective advantage during host colonisation.

The near-universal prevalence of *ompH* (95.9%) and *nanH* (90.5%) agrees with earlier screening panels and likely reflects the essential housekeeping functions these loci serve: *ompH* encodes a porin involved in nutrient uptake and antimicrobial resistance, while *nanH* cleaves sialic acid residues to expose receptors for adhesion [8, 15]. By contrast, *toxA* showed the sharpest serogroup-dependent variation (78.4% in serogroup B versus 11.8% in serogroup D), reinforcing the idea that dermonecrotic toxin production is a feature of the most pathogenic HS clones rather than a conserved species trait [9]. That said, the absence of *toxA* did not preclude isolation from fatal HS cases, suggesting that alternative virulence mechanisms perhaps mediated by iron-acquisition genes like *tbpA* and *hgbB* can compensate when the toxin gene is missing.

From a practical standpoint, these data carry two messages for disease control planners. First, the limited number of dominant clones means that a well-designed bacterin or subunit vaccine targeting the outer-membrane antigens of ST-122 and ST-80 could, in theory, cover the majority of circulating strains. Second, the detection of serogroup D in HS outbreaks argues against the long-standing practice of formulating vaccines exclusively with serogroup B and E strains [16]. Including serogroup D antigens or at least monitoring their frequency would close a gap in regional immunisation programmes.

## Conclusion

This research set out to map the molecular diversity and virulence architecture of *Pasteurella multocida* isolates responsible for haemorrhagic septicaemia in water buffaloes across three provinces of northern Thailand. By coupling multi-locus sequence typing at seven housekeeping loci with PCR-based screening of nine virulence genes, the work generated a detailed molecular portrait of 74 confirmed isolates collected over a 29-month window.

Several findings stand out. First, although serogroup B accounted for the largest share of isolates (41.9%), serogroup D made up nearly a quarter of the collection and was present in outbreaks across all three provinces, challenging the long-held assumption that serogroup D plays a negligible role in bubaline HS. Second, MLST identified 11 sequence types grouped into two main clonal complexes and a handful of singletons. The single most common clone, ST-122, appeared in 28.4% of isolates and was recovered from 10 of the 14 sampled outbreaks, pointing to a widely disseminated lineage. Third, the co-carriage of *tox*A, *ptf*A, and *nan*H showed a strong statistical link with serogroup B and with CC-1 membership, providing a molecular signature that could be useful for risk stratification of field isolates.

On the practical side, the results argue for revisiting regional vaccine formulations. A bacterin built exclusively around serogroup B:2 reference strains may leave buffalo herds partially exposed to serogroup D variants. At a minimum, ongoing molecular surveillance should track the frequency and virulence gene cargo of non-B serogroups. Equally, the portability of MLST data makes it straightforward to compare Thai strains with those from other countries in the Greater Mekong Sub-region, which would be an obvious next step. Several questions remain open. Whether the serogroup D strains identified here possess the same *in-vivo* pathogenicity as their serogroup B counterparts has not been tested experimentally; animal challenge trials would be needed to settle that point. Whole-genome sequencing of the dominant STs would also shed light on the horizontal gene transfer events that appear to have shaped virulence gene distribution. And expanding the sampling frame to include apparently healthy carrier animals could clarify whether the clones detected during outbreaks are also maintained in latent nasopharyngeal reservoirs between seasons. These directions would build on the baseline established here and contribute to a more complete picture of *P. multocida* epidemiology in Southeast Asian buffalo production systems.

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- **Conflict of Interest:** Not available
- **Financial Support:** Not available

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