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Comparative Evaluation of Isolation Methods on the Biophysical Properties of Milk Derived Exosomes from Bovine Subspecies

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ABSTRACT

Background: Milk-derived exosomes (MDEs) are stable, nanosized extracellular vesicles secreted by mammary epithelial cells, encapsulating bioactive proteins, lipids and nucleic acids within a phospholipid bilayer. Their stability and resistance to enzymatic degradation make them promising candidates for targeted delivery systems and nutraceutical applications. This study standardized a robust, reproducible protocol for isolating high-purity MDEs from *Bos indicus* (Sahiwal), *Bos taur*us (Holstein Friesian) and crossbred (Karan Fries) cows and evaluated subspecies-specific variations in their properties.

Methods: Differential ultracentrifugation and precipitation kit based methods using specific polymers were compared for efficiency, yield and preservation of exosomal integrity. Comprehensive characterization comprising transmission electron microscopy, dynamic light scattering, nanoparticle tracking analysis and western blotting for exosomal markers.

Result: Our results demonstrate that differential ultracentrifugation is the superior method for yielding morphologically intact, homogeneous MDEs from large milk volumes, irrespective of the milk source. This precision was crucial, as it allowed us to discern subtle but significant subspecies-specific differences. MDEs from *Bos indicus* exhibited a markedly smaller particle size than those from *Bos taurus* and crossbreds. This distinction is critical because exosome size directly impacts their functional performance and utility. *Bos indicus* MDEs potentially offer enhanced cellular uptake and bioactivity, while larger MDEs from taurine and crossbred cattle may be advantageous for delivering bulkier molecular cargos or modulating immune responses. This work not only validates an optimized isolation technique for large-scale recovery of high-quality MDEs but also highlights bovine subspecies nanostructural traits with implications for précised applications in dairy biotechnology and healthcare. Identification of smaller, potentially more bioavailable MDEs from *Bos indicus* highlights the importance of native cattle resources for high value therapeutic applications, promoting value addition and sustainable utilization of native cattle genetic resources.

Key words: Breeds, Characterization, Isolation, Milk derived exosomes.

INTRODUCTION

Cow milk is one of the world's most consumed foods revered for its optimal balance of essential nutrients that are vital for human growth and development (Haug *et al*. 2007). Apart from maintaining brain-body functions and strength-building effects, milk can prevent and reverse health disorders (García-Martínez *et al*. 2022; Liu *et al*. 2023). Over the past two decades, the scientific exploration of milk has transcended its nutritional significance with transitioning towards recognizing milk and its bioactive compounds as functional foods. The potential of milk cargos (macro and micronutrient) to induce profound physiological effects, thereby exerting a tangible influence on human health and well-being is well studied (Pratelli *et al*., 2024). Efforts to identify and evaluate different bioactive milk compounds, biomolecules in cow milk and to assess their positive attributes for value addition are gaining significant momentum (Mills *et al*., 2011). Exosomes are key bioactive components of milk; known to exert diverse physiological benefits, including immune modulation, gut microbiota regulation, intestinal health and overall growth and development (Li *et al*., 2022). Milk-derived exosomes (MDEs), nanosized extracellular vesicles (~40-160 nm) secreted by mammary epithelial cells, play crucial roles in intercellular communication and can cross biological barriers such as the blood-brain barrier (Théry *et al*., 2018). They carry a rich cargo of proteins, lipids, metabolites and nucleic acids that reflect their cellular origin (Betker *et al*., 2019), enabling them to influence recipient cell physiology.

MDEs possess dual potential acting as natural bioactive molecules and as efficient, biocompatible drug

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delivery vehicles. Dietary MDE supplementation has been associated with enhanced intestinal architecture, immune modulation, skeletal muscle synthesis and anti-inflammatory, antioxidant and anticancer effects (Gao *et al*., 2019). Their uniform size, stability, low immunogenicity and ability to cross biological barriers make them ideal for oral therapeutic delivery (Li *et al*., 2024; Garg *et al*., 2025). India, with over 192 million cattle, possesses rich genetic diversity across *Bos indicus*, crossbred and *Bos taurus* cattle. Many indigenous breeds, though resilient and adapted to tropical conditions, remain underutilized due to lower milk yields. Characterizing breed-specific MDEs offers a novel approach to add value, promote sustainable utilization and conserve these important genetic resources. Isolation of pure, intact exosomes and characterization of their physicochemical properties thereafter is a key for success of MDEs as bio vehicles as these might affect the overall therapeutic properties or delivery efficiency of loaded molecules. The differential response of exosomes derived from milk of different livestock species or subspecies can be attributed to the specific size, structure and biomolecules in their respective cargo (Tauro *et al*., 2012; Rekker *et al*., 2014; Mahajan *et al*., 2025). There might also be changes in the physiochemical properties of MDEs across subspecies. Delineation of source specific characteristics of MDEs adding advantage to their use as therapeutic agents can also add value to the source breeds/ subspecies thus enhancing their utility leading to sustainable conservation. This holds special importance for many of the indicus breeds which are on the verge of extinction due to their low production potential.

Though widely accepted as food adjuncts worldwide, there are some technical challenges associated with isolation, purification of exosomes from the group of extracellular/microvesicles; their accurate characterization because of their small size, low refractive index and heterogeneity. High fat and casein content of milk also affects the purity and yield of MDEs adversely. The implied isolation methods not only influence the exosome morphology (Kona *et al*., 2024) but also the composition of their cargo (miRNA, metabolites and protein *etc*). In this study we have attempted to isolate the exosomes derived from milk of indicine (*Bos indicus*), crossbred (cross of indicus and taurus) and exotic (*Bos taurus*) cows, which are the major milk sources in India (43%) and across the globe (10.75%) (DAHD, 2023-24).This study aimed to standardize the technique for extraction of intact and homogenous exosomes from milk of different cow subspecies, examine their physiochemical properties and assess the differences across subspecies, if any.

MATERIALS AND METHODS

Sample collection and exosome isolation

Ten unpasteurized milk samples (50-60 ml) each of Sahiwal, representative of *Bos indicus* cattle; Karan Fries, a crossbreed (indicus × taurus); and Holstein Friesian, representative of *Bos taurus* cattle were collected from National Diary Research Institute (NDRI, Karnal). The dairy cows used in the study were healthy, non-pregnant multiparous. Diet and environmental conditions were same for all the three cattle types and the samples collected were of mature milk (60 to 90 days). Fresh milk samples were defatted the same day and further processed for isolation of milk exosomes. Exosomes were isolated using differential ultracentrifugation or precipitation kit-based method.

Differential Ultracentrifugation

The protocol of differential ultracentrifugation used in the study is the modified version of Vaswani *et al*. (2017). The procedure included removal of fat, cellular debris by centrifugation at 5000 rpm for 10 min at 4°C. Supernatant was collected without disturbing the pellet and centrifuged firstly at 12,000 × g for 60 min and then at 70,000 × g for 60 min to pellet-out larger vesicles and remaining cellular debris from the matrix. The supernatant obtained was filtered twice through membranes of pore size 0.45 and 0.22 µm respectively to remove larger EVs and apoptotic bodies. Filtration through 0.45 µm pore size membranes was addition step to the original procedure so as to filter large size extracellular vesicles and avoid blockage of 0.22 µm filters. Following this, the filtrate was subjected to centrifugation at 100,000 ×g for 60 min and the resulting pellet was collected. After this, an additional cycle of centrifugation at 120,000 ×g for 60 min was introduced to pellet smaller exosomes. Supernatant was discarded carefully. The pellets obtained from the 100,000 ×g and 120,000 ×g centrifugation steps were washed by resuspension in phosphate-buffered saline (PBS) to remove soluble proteins and contaminants. Following this, the pellets were pooled and resuspended in a final volume of 1 ml PBS. The exosomes preparation was stored at -80°C until further analysis.

Precipitation kit-based method

Isolation of exosomes based on precipitation kit involved all the steps as that of differential ultracentrifugation till filtration through 0.22 µm filters. After filtration through 0.22 µm pore size membranes, the samples were treated with an EQ reagent (250 µl/ 63 ul of filtrate) (#EXOQ5A-1 ExoQuick), mixed gently and incubated for 12 hours at 4°C to facilitate precipitation. Afterwards, centrifugation was carried out at 1500 × g for 30 minutes at 4°C to pellet the exosomes. The supernatant was removed and the pellet was centrifuged at 1500 × g for 5 minutes at 4°C to remove residual ExoQuick solution. The pelleted exosomes were carefully resuspended in 200-400 µl of 1×-filtered PBS (Fig 1).

Characterization of exosomes

The milk derived exosomes isolated using differential ultracentrifugation and the commercial precipitation kit-based methods using specific polymers, were subjected to comprehensive characterization to evaluate their physicochemical properties and molecular specificity.

Morphological examination of the exosomes was performed using transmission electron microscopy (TEM), to confirm vesicle integrity and typical cup-shaped structure. Particle size distribution and concentration were determined using nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). For molecular profiling and to confirm the presence of exosome-specific markers (CD9 and CD81), Western blot analysis was conducted using standard immunodetection protocols.

Western blotting

The experimental procedure for confirming the presence of exosomal specific biomarkers via Western blotting involved the homogenization of exosomal samples using radio immunoprecipitation assay (RIPA) buffer (Himedia TCL131) supplemented with protease inhibitors to maintain protein integrity. Equal volumes of lysates were denatured in reducing buffer and resolved on 4-20% Tris-glycine SDS-PAGE gels, followed by electrotransfer onto PVDF membranes. After blocking with 5% non-fat milk, membranes were incubated with primary antibodies against CD9 (Invitrogen, #MA1-80307) and CD81 (Santa Cruz, #sc-166029) for exosomal marker detection and CD40 (Santa Cruz, #sc-13128) as a control marker. Following TBS-T washes, membranes were treated with HRP-conjugated goat anti-rabbit secondary antibody (#31460) for 1 hour at room temperature. Protein bands were visualized by chemiluminescence using X-ray film, confirming the presence of characteristic exosomal markers.

Transmission electron microscopy

For ultra structural analysis, using transmission electron microscopy (TEM), MDEs were transferred onto Formvar/ carbon-coated copper grids and air-dried at room temperature for 20 minutes. The grids were subsequently rinsed briefly washed and fixed using 1% (w/v) glutaraldehyde in PBS, followed by several washes with distilled water to remove any residual fixative. The samples were then contrasted with 4% (w/v) uranyl acetate (UA) and a UA-methylcellulose solution for 10 minutes on ice to enhance visualization. Imaging was performed using a transmission electron microscope (JEOL JEM-F200), where exosomes were visualized under an electron beam. High-resolution images were captured, providing detailed insights into the morphology and structural characteristics of the exosomes, facilitating thorough analysis and interpretation.

Nanosight tracking analysis (NTA)

The size distribution, mode and concentration of MDEs were analyzed using a NanoSight™ NS300 system (Malvern Instruments, UK) equipped with a 45 mW 405 nm laser and an EMCCD camera. The instrument was calibrated and optimized to track approximately 30 particles per frame, with camera level 12, detection threshold 5 and temperature maintained at 25°C. Exosomal samples were resuspended in PBS and diluted 1:100 to achieve optimal particle visibility. Each sample was infused into the chamber using a syringe pump at a constant flow rate of 40 and three 60 s videos were recorded for analysis. Data

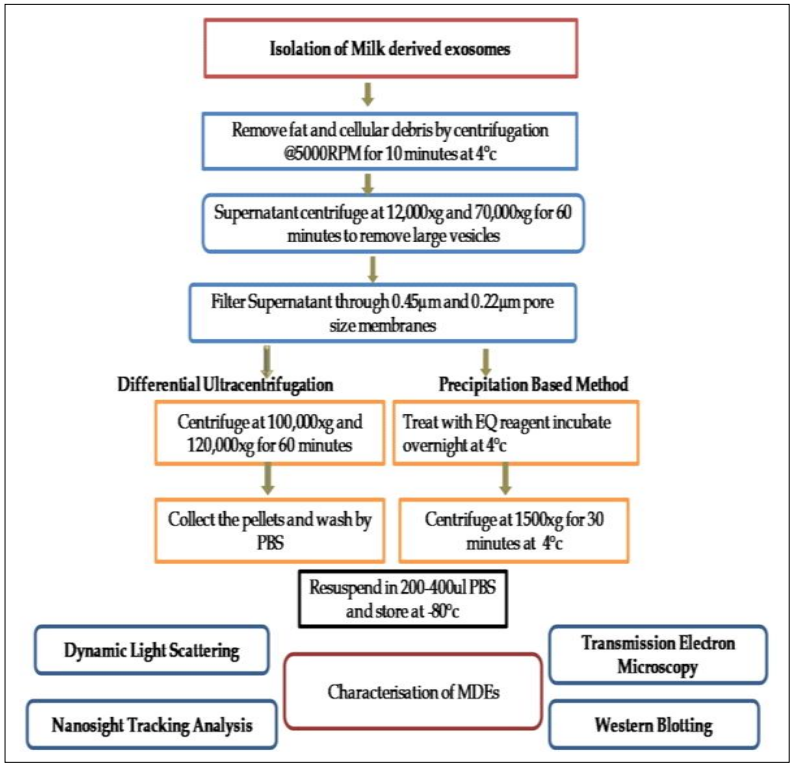


Fig 1: Flowchart representing procedural details for isolation of milk MDEs and physiochemical characterization.

were processed using NTA software (version 2.3.0.17) to determine particle size distribution, mode and concentration. This technique, which tracks the Brownian motion of individual vesicles via light scattering, allows accurate measurement of particles as small as 30 nm, offering a rapid and reliable assessment of MDE characteristics.

Dynamic light scattering (DLS)

For determining the size of exosomes derived from different milk sources, dynamic light scattering (DLS) measures random fluctuations in the intensity of light scattered from exosomal suspensions. For the analysis, exosomal solution diluted in water in the ratio of 1:100 was examined at a temperature of 25°C, with an equilibration time of 120 seconds. A laser beam with a wavelength of 632.8 nm was directed at the exosomal suspension and the scattered light was detected at a 173° angle using an avalanche photodiode detector (APD) and non-invasive back scattering (NIBS) optics (Malvern Instruments Ltd.). Each sample measurement was repeated three times and the average size of the exosomes was calculated from these replicates to ensure accuracy and reliability.

RESULTS AND DISCUSSION

Isolation of exosomes

Exosomes were successfully isolated from the milk of *B. indicus*, *B. taurus* and crossbred cows using two distinct methods: differential ultracentrifugation and a precipitation kit-based method. Differential ultracentrifugation separates exosomes based on size, shape and density through multiple high-speed centrifugation steps, offering higher purity. In contrast, precipitation kit-based method use chemical reagents like ExoQuick to isolate exosomes with simpler procedures, though they may result in lower purity due to co-precipitation of impurities. The efficacy of both methods was evaluated through comprehensive characterization using multiple analytical techniques including transmission

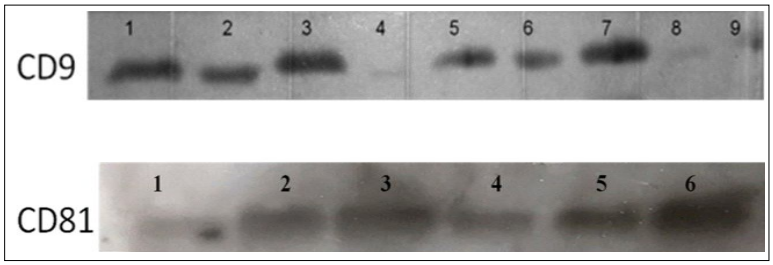


Fig 2: Western blot analysis of MDE isolated from different sources of milk.

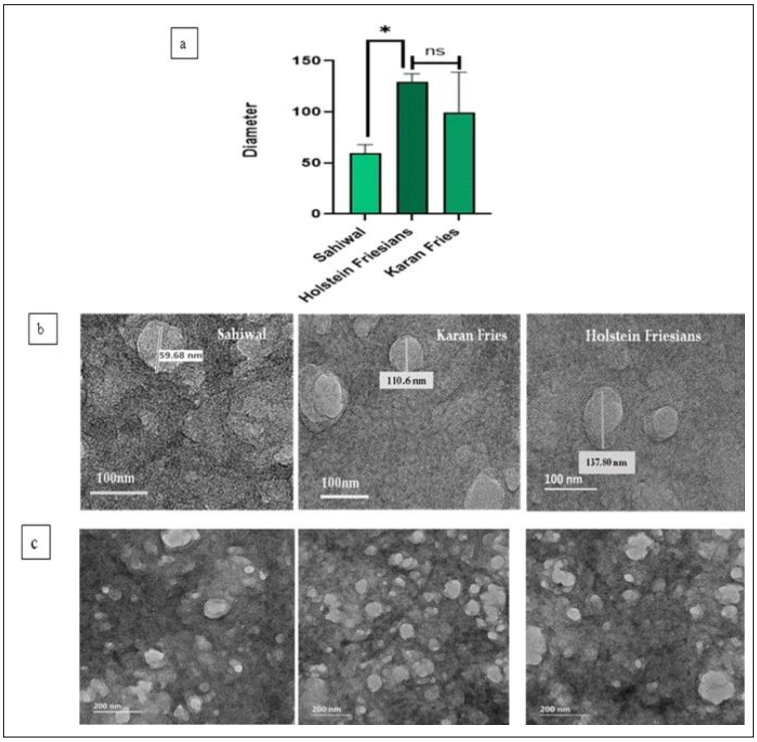


Fig 3: a) Transmission electron microscopy of MDE, depicting vesicles with exosome specific shape, morphology and diameter range across three different milk sources isolated by b) Differential ultracentrifugation and c) Precipitation based method.

electron microscopy (tem), nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). These analyses provided a detailed profile of the yield, purity, particle size distribution, physicochemical properties and marker specificity of the MDEs.

Molecular profiling of exosomal marker proteins

The identity and purity of isolated vesicles were confirmed by immunoblotting for exosome-specific markers CD9 and CD81 and CD40 as a control to check contamination by other multivesicular bodies. Molecular profiling confirmed the presence of distinct and consistent bands for exosome-specific markers CD9 and CD81 at their predicted molecular weights (25-26 kDa) in the all samples (Fig 2). These findings were compared against the PageRuler plus Prestained Protein Ladder, 10 to 250 kDa, as a molecular weight reference. Importantly, no bands were detected for the control marker CD40, which is associated with other multivesicular bodies, confirming the high purity of the isolated exosome population. Further, no exosomal markers were observed in the somatic cell control group, reaffirming the specificity of the isolation methods. Specific protein marker signals were observed for the isolated MDEs, regardless of the isolation method or milk source/ cattle type, demonstrating successful isolation (Fig 2).

Morphological characterization using transmission electron microscopy

Transmission electron microscopy (TEM) indicated that exosomes isolated from all three milk sources and different isolations methods exhibited the characteristic spherical shape and bilayer morphology. Though the overall morphology was similar, some differences were observed for the method of isolation used or the source of milk (Fig 3a).

Differential Ultracentrifugation derived exosomes exhibited well-defined, homogeneous spherical morphology, with a consistent vesicle diameter ranging from 53 to 137 nm (Fig 3b). In contrast, the precipitation kit method resulted in a more heterogeneous population, with visible aggregation and larger vesicles exceeding 200 nm in diameter (Fig 3c). Breed-specific size variations were also observed. MDEs from Sahiwal, Karan Fries and Holstein Friesian averaged 62.52 nm, 77.58 nm and 129.2 nm, respectively. The average MDEs size derived from indicine and taurine breeds differed significantly (Table 1), whereas no significant differences were observed for MDE size derived from crossbred and exotic breeds.

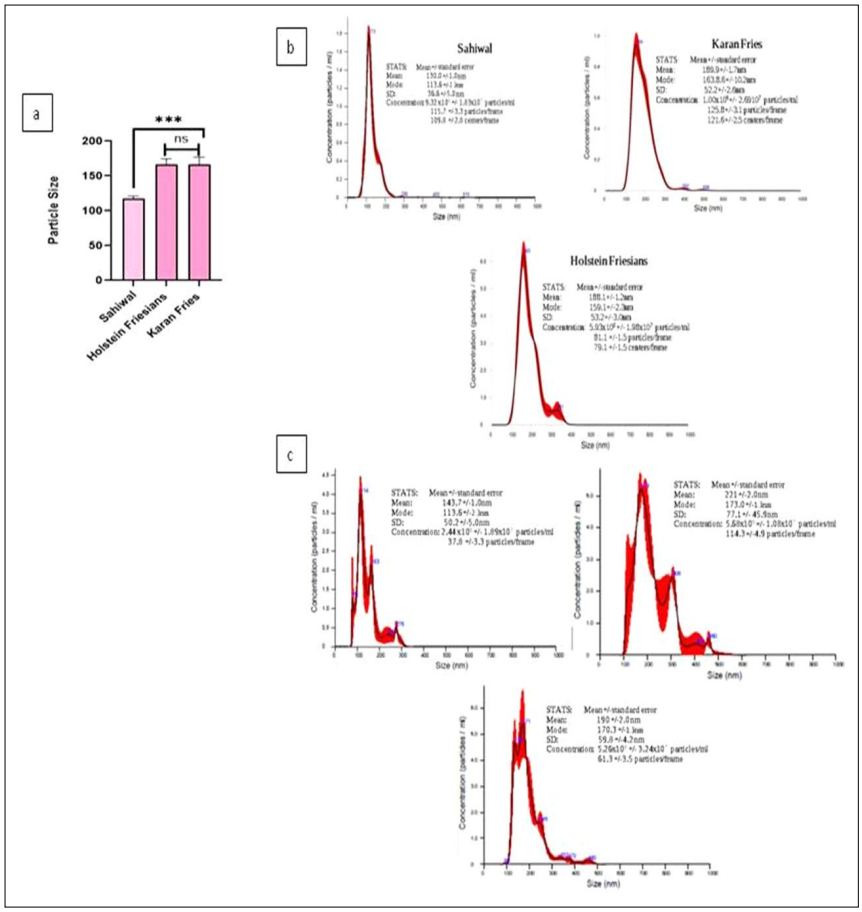


Fig 4: a) Nanoparticle tracking analysis (NTA) to determine the size distribution and particle concentration (particles/ml) of exosomes isolated using b) Differential ultracentrifugation and c) Precipitation Kit-based method across different milk sources.

Particle size and concentration analysis using NTA

Nanoparticle tracking analysis (NTA) provided quantitative data on MDEs size and concentration. MDEs isolated by differential ultracentrifugation showed a narrow size distribution and superior specificity, as reflected by consistent particle size and concentration measurements across different sources. For the *Bos indicus* (Sahiwal) MDEs, the average particle size was 117.27 nm, with a particle concentration of 111.83±4.5 particles per frame. Exosomes derived from the milk of crossbreed (Karan Fries) and taurine breeds (Holstein Friesian), had the average particle sizes of 166.6 nm and 166.53 nm, respectively. The Sahiwal MDEs were significantly smaller than the Karan Fries and Holstein Friesian MDEs. However, difference in the MDEs particle size between the taurine and crossbreed cattle were not significant, indicating comparable MDEs characteristics for these two subspecies. The average particle concentrations extrapolating to the starting volume of per ml milk used were 8.58×10^8, 7.75×10^8 and 8.08×10^8 particles/mL for Sahiwal, Karan Fries and Holstein Friesian, respectively (Fig 4b). These findings highlight significant breed-specific variations in MDEs particularly between indicus *vis* a *vis* exotic/crossbreed cattle (Fig 4a).

In contrast, MDEs isolated using the precipitation method displayed a broader, more variable size distribution, with multiple size peaks indicative of aggregation (Fig 4c). The average particle concentration was significantly lower (4.46×10^8 particles/ml), suggesting reduced yield and purity due to co-precipitation of non-exosomal particles. Due to the high variability in particle size breed specific differences were not apparent. The concentration of particles also indicating high aggregation and variability with an average of 4.46×10^8 particles/mL, varied significantly across milk sources and was significantly lower than differential ultracentrifugation based isolation, suggesting reduced yield and purity due to co-precipitation and aggregation of particles (Table 1).

Particle size and concentration analysis using DLS

Dynamic Light Scattering (DLS) corroborated the findings of TEM and NTA. Exosomes isolated *via* differential ultracentrifugation showed consistent Z-average sizes and low polydispersity index (PDI). The Z-average sizes for exosomes from the indicine breed were observed at 67.91, 97.98 and 72.06 d.nm with PDIs of 0.308, 0.243 and 0.217, respectively. Crossbred exosomes exhibited sizes of 119.0, 121.9 and 146.0 d.nm, with corresponding PDIs of 0.416, 0.445 and 0.422 (Table 1). Exotic breed exosomes

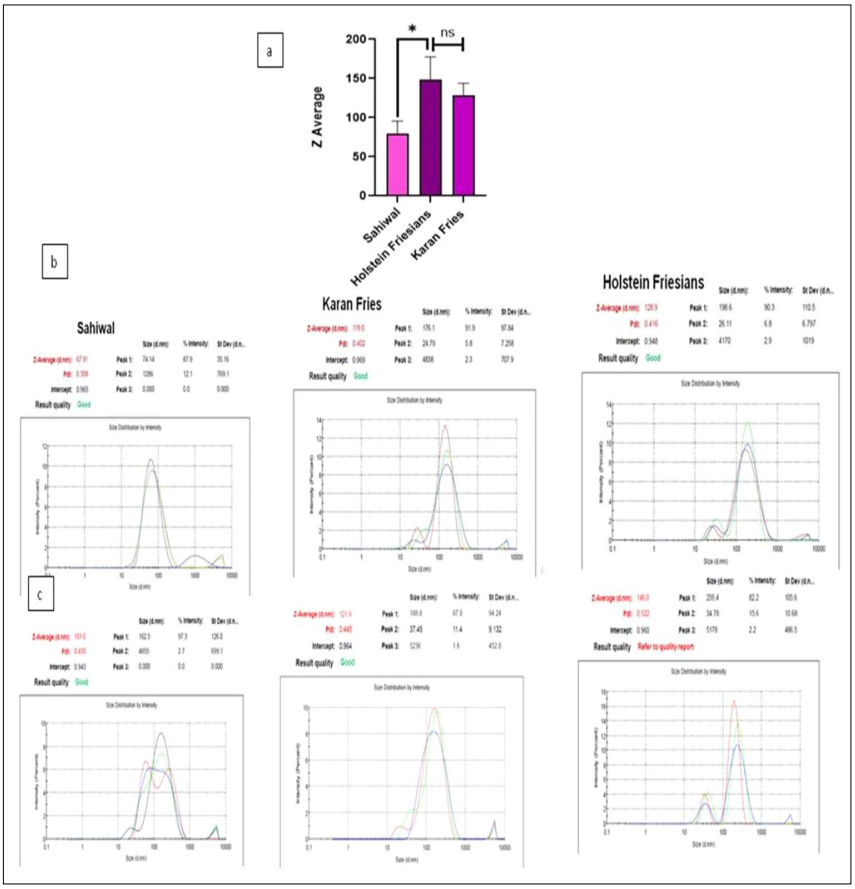


Fig 5: a) Particle size distribution of MDEs across indicine, crossbreed and exotic cattle breeds isolated using b) Differential ultracentrifugation method c) Precipitation kit based method.

displayed Z-average sizes of 128.9, 136.1 and 181.7 d.nm with PDIs of 0.402, 0.289 and 0.249 respectively (Fig 5b, Table 1). Comparative analysis revealed no significant differences in exosome size distribution between crossbred and exotic breeds. However, the Z-average of Sahiwal was significantly lower compared to Holstein Friesians and Karan Fries (Fig 5a).

In contrast, the precipitation-based method failed to discern such subtle differences due to the broad size distribution. Multiple size distribution peaks were observed corresponding to diameters exceeding 5000d.nm, along with PDI values above 0.5 (Fig 5c), indicative of highly heterogeneous population and likely contamination. The broad distribution curve, coupled with higher standard deviations for peak sizes, underscores the reduced specificity of the precipitation-based method in isolating pure exosome populations.

Milk is a functional food that supports growth and development while serving as a rich source of bioactive molecules with health-promoting properties. Among these, exosomes nanoscale vesicles secreted by mammary epithelial cells play critical roles in intercellular communication, immune modulation, tissue repair and disease regulation (Rashidi *et al*., 2022). Bovine MDEs have gained particular interest due to their scalability, high biocompatibility and low immunogenicity, enabling their potential in nutritional and therapeutic applications (García-Martínez *et al*., 2022; Zhang *et al*., 2023; Jan *et al*., 2019; Théry *et al*., 2018). However, isolation of pure, intact MDEs remains challenging due to the complex milk matrix containing casein micelles, fat globules and other vesicles (Wu *et al*., 2019). Despite several available techniques (Van Herwijnen *et al*., 2018), no standardized subspecies-specific protocol yet exists. The present study compared the efficacy of two commonly used isolation techniques that is differential ultracentrifugation and precipitation kit based using specific polymers to isolate MDEs from three major bovine subspecies (breeds): *Bos indicus* (Sahiwal), *Bos taurus* (Holstein Friesian) and their crossbreds (Karan Fries). These three subspecies were selected as they are the major contributor for cow milk across globe and in India (Department of Animal Husbandry and Dairying, 2024).

Differential ultracentrifugation emerged as the most effective method for isolating MDEs, offering superior specificity, homogeneity and reproducibility despite being time-intensive. Sequential centrifugation combined with double filtration (0.45 µm) efficiently removed fat globules, casein and larger vesicles, yielding a highly pure and uniform exosomal population. The final ultracentrifugation step (100,000×g-120,000×g) significantly enriched the exosomal fraction. Transmission electron microscopy (TEM) revealed characteristic spherical or “doughnut-shaped” vesicles, while nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) confirmed narrow size distributions and low polydispersity index (PDI) values. In contrast, the precipitation kit-based method, though faster and simpler, produced heterogeneous vesicle populations with lower purity and aggregation, limiting its suitability for sensitive downstream analyses. These findings align with earlier reports highlighting the reduced specificity of polymer-based precipitation methods (Mehdiani *et al*., 2015). While ultracentrifugation is time-demanding, its scalability and reproducibility make it a reliable choice for routine and large-scale MDE isolation.

Differential ultracentrifuge proved to be a more sensitive method and could discern the size differences in exosomal derived from three milk sources. It revealed significant size differences between Indicine vis a vis taurine/crossbred cattle MDEs while the size differences between taurine/crossbred cattle were nonsignificant. Overall, *B. indicus* (Sahiwal) yielded significantly smaller exosomes (~79.3 nm) compared to *B. taurus* and crossbreds. These findings highlighted the sensitivity of ultracentrifugation in capturing breed-specific variations.

Table 1: Comparative biophysical profiles of MDEs sourced from cows belonging to different subspecies and isolated using differential ultracentrifugation and precipitation based kit methods.

Biophysical characteristics	Method of isolation	Sahiwal	Karan fries	Holstein friesians
Particle size	Ultracentrifugation	113.0 nm	163.8 nm	159.1 nm
	Kit-Based	113.6 nm	173 nm	170.3 nm
Particles concentration (particles/ml)	Ultracentrifugation	9.32e+008± 1.83+007 particles/ml	1.00+009± 2.69+007 particles/ml	5.93+008± 1.98+007 particles/ml
	Kit-Based	2.44e+008± 1.89+007 particles/ml	5.68+008± 1.08 +007 particles/ml	5.26+008± 3.24+007 particles/ml
Z-average	Ultracentrifugation	67.91 d.nm	119.0 d.nm	128.9 d.nm
	Kit-Based	101 d.nm	121.9 d.nm	146.0 d.nm
Polydisperity index	Ultracentrifugation	0.30	0.40	0.41
	Kit-Based	0.43	0.44	0.52
Diameter	Ultracentrifugation	53 nm	110.6 nm	137.80 nm
	Kit-Based	not scalable	not scalable	not scalable

This breed-specific distinction was not evident using the precipitation method, which produced high heterogeneity in particle size distribution or inconsistent size measurements due to vesicle aggregation and poor resolution as revealed by multiple particle size peaks.

DLS results also supported the superiority of the differential ultracentrifugation technique. The Z-Average size range (67-129 nm) and PDI <0.45 confirmed the homogeneity of exosomes isolated *via* differential ultracentrifugation. These values align with previously reported size ranges for MDEs from various livestock species *e.g.*, yak (~131 nm Gao, 2019), cow (~140 nm Vaswani, 2017) and bovine (~131.5 nm) Z-average of Sahiwal MDEs was significantly lower (79.3 nm) compared to Holstein Friesians and Karan Fries indicating well-dispersed exosome populations without significant aggregation and with minimal intensity. In contrast, exosomes isolated using the precipitation-based method exhibited a broader size distribution with evidence of particle aggregation (PDI >0.5) and higher aggregations of vesicles in the samples. This study not only validates ultracen-trifugation as a robust method for isolating high-quality MDEs across cattle sub species/ breeds but also highlights breed-specific differences in exosomal size and concentration particularly the smaller, more uniform exosomes from *B. indicus* (Sahiwal) compared to B. taurus and crossbreds. This distinction, which was only discernible with the precision of the differential ultracentrifugation method, has important implications.

This distinction holds important as size and heterogeneity of MDEs are crucial factors that significantly influence their efficacy and utility across various different applications. It impacts the exosome cargo composition (protein, nucleic acids and RNA profiles; (Menck *et al*., 2020) in turn influences their biological functions and therapeutic potential when MDEs used as such as supplementary food. Size is also an important factor when MDEs are used as nanoparticle carriers, influencing their biological functions, cargo composition, cellular uptake, stability and therapeutic efficacy.

Smaller exosomes, such as those from *Bos indicus*, may have distinct advantages including enhanced membrane penetration and potentially longer circulation times, making them more effective as nanocarriers for targeted delivery of bioactive molecules. The size of exosomes can influence their interaction with target cells, affecting how they deliver their cargo. Smaller exosomes may be internalized readily (Sharma *et al*., 2024). In contrast, larger vesicles may be more effective in delivering larger cargoes including proteins, nucleic acids and may be more effective at mediating specific signaling pathways (Kowal *et al*., 2014). Size variations also influence exosome stability in biological fluids and smaller exosomes may have better stability (Xia *et al*., 2024). In Immune Modulation different sizes of exosomes can elicit varying immune responses, with larger exosomes potentially carrying more immunomodulatory factors. In the context of diseases like cancer, size variations in exosomes can influence the types of oncogenic factors present, impacting disease progression and response to therapy (Menck, 2020), when consumed as such and also or as nano-carriers for targeted delivery.

Taken together, these physiochemical properties are especially relevant in translational medicine, where exosomes are being investigated as drug delivery vehicles, immune modulators and even diagnostic biomarkers. The observed differences in MDEs size between breeds underscore the importance of selecting appropriate exosome sources and isolation methods for specific biomedical applications. The reliable isolation of pure MDEs also enables the comparisons of miRNA, metabolome, proteome or lipidomic profiles across the different cattle types as well between human MDEs (Koh *et al*., 2017; Vaswani *et al*., 2019; Garg *et al*., 2025). Therefore, the isolation of high-quality exosomes is essential for advancing exosome research.

CONCLUSION

In conclusion, our comprehensive characterization highlights the importance of choosing an appropriate isolation method to ensure the purity and integrity of MDEs. The data strongly support differential ultracentrifugation as the highly effective method capable of isolating high-quality, intact, homogeneous and biologically relevant exosome populations in high concentrations suitable for downstream applications irrespective of cow subspecies used as milk source. This study not only validates a robust isolation technique but also uncovers significant physiochemical differences in MDEs from different bovine subspecies. Confirmation of fact that exosomes derived from milk of *Bos indicus* cows are smaller in size ensures their upper edge to be used as therapeutic carriers for delivery of bioactive molecules. These findings add value to the utilization of *Bos indicus* milk and its derivatives, supporting its sustainable utilization and conservation.

The ability to isolate pure exosomes with consistent biophysical profiles not only enhances their utility as therapeutic carriers but also supports advanced omics-based evaluations (e.g., miRNA, proteome, metabolome, lipidome) and functional studies across bovine breeds and between species, including humans to harness the full potential of MDEs in human and animal health.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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