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# Comparison of exosomes purified via ultracentrifugation (UC) and Total Exosome Isolation (TEI) reagent from the serum of Marek's disease virus (MDV)-vaccinated and tumor-bearing chickens

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ABSTRACT

Extracellular vesicles (EVs) is a collective term used to refer microparticles, exosomes, and apoptotic bodies produced by a variety of cells and released into interstitial spaces and bodily fluids. Serum exosomes can serve as invaluable biomarkers, containing m/miRNAs, lipids, and proteins, indicative of various conditions. There are currently limited studies on the characterization and mutual consensus of biomarker profiles of serum exosomes purified by different methods. Here we compared the advantages and disadvantages of two commonly used serum exosome purification procedures including ultracentrifugation (UC) and Total Exosome Isolation (TEI) reagent, by analyzing exosome size distribution, concentration, morphology and miRNA expression profiles. Serum was obtained from Marek's disease virus (MDV)-infected chickens that were either vaccinated against Marek's disease (MD), and thus protected, or unvaccinated and bearing MDV-induced tumors. Nanoparticle tracking analysis (NTA) and Transmission Electron Microscopy (TEM) were performed to evaluate particle size, concentration, and morphological integrity, respectively. Our results indicate that the size distribution of particles purified by either procedure is consistent with that of exosomes (30–150 nm). TEI reagent generated higher yields and co-isolated additional EV populations that are slightly larger (~180 nm). Based on the miRNA expression profiles from a previous high throughput sequencing experiment of exosome small RNAs, we selected six cellular and four MDV1 miRNAs, to validate their expression in UC- and TEI-purified exosomes. miRNA expression profiles displayed relative correlation between the two procedures, but distinctive differences were observed in abundance with TEI-purified exosomes showing higher miRNA expression consistent with higher yield than those purified by UC. TEI-purified exosomes from vaccinated chickens exhibited greater expression of tumor suppressor miRNA, gga-mir-146b and least expression of oncomiR, gga-mir-21 compared to those obtained from tumor-bearing chickens. We propose that gga-mir-146 and -21 can serve as serum exosome biomarkers for vaccine-induced protection and MD tumors respectively.

## 1. Introduction

Extracellular vesicles (EVs) are phospholipid bilayer-enclosed, spherical particles released by a variety of cell types into biological fluids such as blood, urine, breast milk, bile, bronchoalveolar lavage, genital, cerebrospinal, ascitic and amniotic fluids, as well as by cultured cells *in vitro* (Admyre et al., 2007; Alegre et al., 2014; Conde-Vancells et al., 2008; Hegmans et al., 2004; Neerukonda et al., 2017; Nilsson et al., 2009; Rabinowits et al., 2009). Based on their size, origin, and biogenesis, they are categorized into microvesicles, exosomes and

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apoptotic bodies (Lawson et al., 2016).

Microvesicles range in size from 100 to 1000 nm and directly bud from the plasma membrane (PM) (Yanez-Mo et al., 2015). Exosomes range in size from 30 to 150 nm and originate in late endosomes or multi-vesicular bodies (MVBs) upon inward invagination of the endosomal-limiting membrane. MVB fusion with the PM leads to the release of exosomes into the extracellular space (Yanez-Mo et al., 2015). Apoptotic bodies range in size between 50–5000 nm and originate from cells undergoing apoptosis via a blebbing mechanism (Yanez-Mo et al., 2015).

Among EVs, exosomes are considered crucial vehicles for intercellular communication as they carry functionally active messenger RNAs (mRNAs), micro RNAs (miRNAs), proteins, and lipids between cells to mediate a range of biological effects upon target cell binding and uptake (Yanez-Mo et al., 2015). The ease of collection of biological fluids (e.g. blood, urine), and capacity of exosomes to reflect the physiological or pathological state of the originating cell, led to the proposition that exosomal miRNAs and/or proteins can serve as excellent biomarkers for disease diagnosis or prognosis (Ailawadi et al., 2015; Yanez-Mo et al., 2015; Zhao et al., 2015). Amid growing enthusiasm in utilizing exosomes for biomarker identification and discovery, there lacks a technical standardization in the procedures employed to purify and analyze EVs, including exosomes (Witwer et al., 2013). The influence of various procedures on exosome size, integrity, and recovery, and its effect on their RNA and protein content remains unclear. Hence, there is a need to provide a definition of “best practices” and standardization of exosome purification procedures (Witwer et al., 2013).

Current exosome purification procedures in use include classical differential ultracentrifugation (UC) (Théry et al., 2002), density gradient UC (sucrose/iodixanol) (Lobb et al., 2015; Théry et al., 2006), size exclusion chromatography (SEC) (Lobb et al., 2015), ultrafiltration (Merchant et al., 2010), marker-based immune affinity isolation (Nakai et al., 2016), microfluidic devices (Contreras-Naranjo et al., 2017), commercial polymer-based precipitation reagent kits (ExoQuick™, System Biosciences), Total Exosome Isolation reagent (TEI, Invitrogen), miRCURY (Qiagen) (Alvarez, 2014; Cheng and Schorey, 2013; Kadiu et al., 2012) and volume excluding polymers (Polyethylene Glycol [PEG], dextran and polyvinyls) (Rider et al., 2016).

For further enrichment of exosome fractions from culture or biological fluids, paired combinations of aforementioned methods have been employed including microfiltration paired with UC, UC paired with density gradient UC, and PEG paired with an UC wash (Rider et al., 2016). Among above procedures, UC is the conventional gold standard procedure which is technically-laborious, time-consuming, requires special equipment and training, making it unsuitable for use in a routine diagnostic laboratory (Ding et al., 2018; Théry et al., 2002).

Alternatively, commercial precipitation reagent kits currently available offer quick and easy procedures that require low input sample with no specialized equipment or training (Peterson et al., 2015). Furthermore, a handful of studies evaluated the qualitative and quantitative performance of UC versus various commercial precipitation reagent kits, in terms of exosome recovery and downstream miRNA/protein expression from human serum (Alvarez et al., 2012; Ding et al., 2018; Helwa et al., 2017; Rekker et al., 2014; Royo et al., 2016a, b; Saenz-Cuesta et al., 2015). These studies reported superior exosome recovery and greater exosomal miRNA and/or protein content by the commercial precipitation reagent kits compared to traditional UC. On the other hand, no study has thus far evaluated the performance of a commercial exosome precipitation reagent kit in comparison to UC in terms of exosome recovery from animal serum.

In the present work, we performed a comparative study of exosome purification procedures, UC versus TEI reagent, to determine to what extent the selected exosome purification procedure influenced size, concentration, integrity and miRNA content of serum exosomes from Marek’s disease virus-infected chickens.

Marek’s disease virus (MDV) is an acute transforming alphaherpesvirus that causes Marek’s disease (MD) in infected chickens. MD is characterized as a paralytic, inflammatory, and immune-suppressive disease, most notably for the rapid development of visceral and peripheral T-cell lymphomas (Parcells and Burgess, 2008). MD is controlled in commercially-raised poultry through the application of non-sterilizing, cell-associated vaccines. Among currently licensed MD vaccines for use in the US, an attenuated MDV1 derivative, CVI988/Rispens, is the most antigenically related to pathogenic MDV1 field strains, and thus provides a high level of vaccinal protection (Spatz et al., 2007). CVI988/Rispens is currently considered ‘the gold standard’ among MD vaccines.

Although losses due to MD are currently controlled by vaccination, MD remains a threat to poultry production due to vaccination costs, vaccine failure, carcass condemnation at processing, and the evolution of field strains of MDV towards higher virulence (Padhi and Parcells, 2016). In this regard, presently no diagnostic biomarkers exist that allow a successful detection of MD vaccine-mediated protection versus disease progression in MDV-infected chickens.

From a previous independent MD challenge study, by employing the Illumina high-throughput sequencing platform, we identified significant and differentially-expressed (SDE) exosomal miRNAs in the serum of CVI988-vaccinated and protected leghorns, and unvaccinated leghorns that were found to be tumor-bearing. In the present study, we validated the expression of (6) domestic chicken (*G. gallus*, gga-) and (4) MDV1(MDV1-) SDE miRNAs, selected based on their expression ranging from low (gga-mir-21, MDV1-mir-M4, -M12, -M6 and –M8) to high (gga-mir-146b, -10b, -2188, -27b, and -99a) in exosomes purified from CVI988-vaccinated and protected leghorn sera referred to as “Vaccinate Exosomes” (VEX) compared to exosomes purified from MD tumor-bearing unvaccinated leghorn sera referred to as “Tumor Exosomes” (TEX).

Sera obtained from these, as well as tumor-bearing broiler chickens from another study were subjected to UC and TEI reagent kit exosome purification procedures. Purified exosomes were compared according to particle size, concentration, morphology, and miRNA content.

## 2. Materials and methods

### 2.1. Serum sample collection

Serum exosomes were purified from commercial broiler chickens used in a vaccine trial. Specific samples are detailed in Table 1. Essentially, commercial broilers were either inoculated on the day of hatch with the TK2a-strain of virus (Shedders), and chickens vaccinated at one day-of-age with a 1X commercial dose (~3500 PFU) of a CVI988 (Rispens) vaccine and placed in contact with two-week old, MDV-inoculated (vv + MDV, strain TK2a-inoculated) “shedder” chickens, which had been inoculated at hatch (344 PFU ± 39 PFU/bird) as described previously (Neerukonda et al., 2018, 2016; Tavlarides-Hontz et al., 2009).

**Table 1**
Source of Serum[a] Exosome Samples.

| Bird Tag | Treatment Group | MD Status & sex | miRNA | NTA | TEM |
|---|---|---|---|---|---|
| BL4254 | CVI988-vaccinate | Neg. (M) | + | – | – |
| BL4294 | CVI988-vaccinate | Neg. (F) | + | – | – |
| BL4350 | CVI988-vaccinate | Neg. (M) | + | + | + |
| OR2107 | Inoculated Shedder | + (F, spleen tumor) | + | – | – |
| OR2232 | Inoculated Shedder | + (F, spleen tumor) | + | – | – |
| OR2250 | Inoculated Shedder | + (M, heart tumor) | + | + | + |

+/- – Serum exosomes obtained upon each procedure was either subjected to (+) or not subjected to (-) characterization by the indicated method.

[a] Serum was obtained from commercial broiler chickens, provided as embryonated eggs by Mountaire Farms, Inc., Millsboro, DE used in a vaccine study comparing CVI988 vaccines.

At cull (MD+, MDV-inoculated chickens) or necropsy (vaccinated and protected chickens), whole blood was collected via cardiac puncture using a 10 cc syringe with 18-gauge needle with no anticoagulant. For obtaining sera, syringes were stored at 37 °C for 1 h and then left at 4 °C overnight. Serum samples collected and stored at -80 °C until processed for exosome purification, as described below. The vaccine efficacy study was approved under IACUC protocol #64R-2016-0, addendum 1 and USDA APHIS permit # 130,630.

### 2.2. Exosome purification

Exosome purification was carried out by Ultracentrifugation (UC) and Total Exosome Isolation (TEI) precipitation solution procedures. In order to avoid miRNA expression differences due to inter-bird variations, serum samples from the same bird were used for both exosome purification procedures (see Table 1).

#### 2.2.1. Ultracentrifugation (UC)

Ultracentrifugation was carried out as described by Théry et al. (Théry et al., 2006). Exosome fraction from 1 mL serum was purified by four consecutive centrifugation steps. Serum was diluted with equal volume of PBS and centrifuged at 300 × *g* for 10 min followed by 2000 × *g* for 30 min to pellet cells and cell debris, respectively. Next, a centrifugation step was performed at 12,000 × *g* for 45 min to pellet microvesicles, followed by the transfer of supernatants to 1.5 mL polyallomer ultracentrifuge tubes (Beckman Coulter, Fullerton, CA). Ultracentrifugation was performed for an hour at 110,000 × *g* (Beckman Coulter Optima MAX, TLA-55 fixed angle rotor, *k-factor* 66). Exosome pellets were re-suspended and washed in 1 mL PBS and the ultracentrifugation step was repeated. All centrifugation steps were performed at 4 °C. Final exosome pellets were re-suspended in 1X PBS and aliquots were stored at −80 °C.

#### 2.2.2. Total exosome isolation precipitation (TEI)

Exosomes were purified from 200 µl of serum using TEI reagent (Invitrogen) according to manufacturer's recommendations. Briefly, 1/5 vol of TEI reagent was combined with serum and incubated at 4 °C for 30 min. The mixture was centrifuged at 10,000 × g for 10 min at room temperature. Supernatants were aspirated and exosome pellets were re-suspended in 1X PBS.

### 2.3. Nanoparticle tracking analysis (NTA)

Concentration, mean size, and size distribution profile of particles purified via UC or TEI reagent were evaluated using a Nanosight NS300 instrument (Malvern, Worcestershire, UK) and analyzed with NTA 3.2 Dev Build 3.2.16 software. The following post-acquisition analysis settings were selected: minimum detection threshold 4, automatic blur, and automatic minimum expected particle size. Samples were diluted 1:20 (TEI) or 1:100 (UC) in PBS to obtain concentration profiles directly comparable between particles purified from 200 µl input serum (TEI) versus 1 mL input serum (UC). This dilution strategy allowed us to achieve measured mean particle concentration 0.6–4 × $10^9$/mL. For each sample, five 1 min videos were recorded and analyzed in batch processing mode. Videos were recorded at camera level 9 with minimum expected particle size, track length, and blur setting, all set to default.

### 2.4. Transmission Electron Microscopy (TEM)

For TEM analyses of exosomes, 400 mesh, carbon-coated copper grids were glow discharged with a PELCO easiGlow™ glow discharge system to render the surface of the grids hydrophilic. Grids were briefly floated on drops of purified exosome-PBS suspensions, washed on drops of water, and then negative stained with 2% uranyl acetate. TEI reagent purified exosomes were diluted 1:10 before adsorption to prevent vesicle overcrowding and allow greater resolution. Air-dried grids were examined with a Zeiss Libra 120 transmission electron microscope at 120 kV, and images were acquired with a GatanUltrascan 1000 2k x 2k CCD camera in the bioimaging core at the Delaware Biotechnology Institute at the University of Delaware. For each set of analyses, at least 20 fields were imaged of exosomes purified by each method, representative images are shown.

### 2.5. Exosomal RNA isolation, reverse transcription and qRT-PCR analysis

For total RNA isolation, 100 µl of exosome-PBS suspensions were combined with 1 mL Trizol reagent (Invitrogen™) and total RNA (m/miRNAs) extraction was carried out according to manufacturer's recommendations. Total RNA isolated via Trizol procedure was DNase treated (Ambion) before polyadenylation and reverse transcription (90 min at 37 °C) with an Oligo-dT primer that contained a universal tag on its 5′ end (Universal mir-RT; Qiagen Inc.,) (Zeka et al., 2015). For qRT-PCR expression analyses of miRNAs, cDNAs were diluted 1:4 and subjected to a first PCR cycle with a forward primer specific to the miRNA of interest. Subsequent PCR cycles were carried out by the miRNA specific forward primer and a reverse primer spanning the universal tag (see Table 2).

Exosomal miRNA expression in VEX relative to TEX was calculated upon normalization to the global geometric means of $C_t$ values. Reaction conditions included 1 µl of the diluted cDNA in a 20 µl total reaction volume consisting of 10 µl iTaq SYBR® Green Supermix (Bio-Rad, Hercules, CA), 8.2 µl of nuclease-free water and 0.4 µl (250 nM) of each forward and reverse primer. All reactions, including no template controls and no reverse transcription controls, were performed in duplicates. Cycling conditions included a PCR activation step (15 min at 95 °C) followed by 40 cycles of denaturation (15 s at 94 °C), annealing (30 s at 55 °C) and extension (30 s at 70 °C). Following amplification, melt curve analysis was performed at temperatures ranging from 55 to 95 °C in 0.5°C increments (81 cycles) for 2–3 sec per cycle to confirm

**Table 2**
miRNA Quantification Primers.

| m/miRNA | Forward primer sequence | reverse primer sequence |
|---|---|---|
| gga-miR-2188-5p | AAGGTCCAACCTCACATGTCCT | – |
| gga-miR-10b-5p | TACCCTGTAGAACCGAATTT GT | – |
| gga-miR-99a-5p | AACCCGTAGATCCGATCTTGTG | – |
| gga-miR-146b-5p | TGAGAACTGAATTCCATAGGCG | – |
| gga-miR-27b-3p | TTCACAGTGGCTAAGTTCTGC | – |
| gga-miR-21-5p | TAGCTTATCAGACTGATGTTGA | – |
| MDV1-mir-M12-3p | TGCATAATACGGAGGGTTCT (meq cluster) | – |
| MDV1-mir-M4-5p | TTAATGCTGTATCGGAACCCTTC (meq cluster) | – |
| MDV1-mir-M6-5p | TCTGTTGTTCCGTAGTGTTCTC (LAT cluster) | – |
| MDV1-mir-M8-5p | TATTGTTCTGTGGTTGGTTTCG (LAT cluster) | – |
| Universal miR-RT primer | – | CGTCAGATGTCCGAGTAGAGGTTTTTTTTTTTTTTT |
| Universal reverse primer | – | CCTCTACTCGGACATCTGACG |

lack of non-specific amplification. Primer sequences are provided in Table 2.

### 2.6. Data analysis

For statistical analyses, MS Excel and GraphPad Prism 5 (GraphPad Software, CA, USA) were used. miRNA $C_t$ values were presented as $C_t$ means ± SD. To compare significant differences in miRNA $C_t$ values between VEX and TEX, an unpaired *t*-test was used. For correlation analyses between purification methods, the Pearson's correlation coefficient was calculated. The significance threshold was set to a fold change ≥ 2 with a $p\ value \leq 0.05$.

## 3. Results

### 3.1. Comparison of size, yield and integrity of particles purified by UC and TEI reagent kit

Size, size distribution, and concentration profile of particles purified by UC and TEI reagent were quantified through NTA. The size distribution profile of particles purified by either UC or TEI reagent fell within the anticipated exosome size range of 30–150 nm. Mean diameters of UC-purified particles were 106.3 ± 46.3 nm and 105.6 ± 41.9 nm for VEX and TEX, respectively with an overall mean of 106 nm (Fig. 1A). TEI reagent-purified particles displayed slightly higher mean diameter of 121 ± 57.1 nm and 184.1 ± 50.9 nm for VEX and TEX, respectively with an overall mean diameter of 152.6 nm. The diameter of a majority of UC-purified particles was ca. 73 and 74.9 nm for VEX and TEX, respectively, whereas a majority of TEI reagent-purified particles had a diameter of 77.6 and 181.1 nm for VEX and TEX, respectively (Fig. 1B, 1C, 1D, 1E). Overall, TEI reagent-purified particles displayed significantly higher overall size compared to UC-purified particles. Particle concentrations in UC- and TEI reagent-purified fractions fell within the range of 0.6–1 and 2–4 billion per mL of serum, respectively. Although the TEI reagent yielded slightly higher number of particles/mL, this difference was not statistically different (Fig. 1F).

### 3.2. Visualization of exosome morphology by TEM

Upon finding that the diameters of particles purified by both the procedures fell within the expected size range of exosomes, we further confirmed the morphological integrity of purified particles via TEM, a well-accepted technique for nanoparticle validation. Negative staining of VEX and TEX particles purified by each procedure displayed typical spherical morphology within the anticipated size range of exosomes (Fig. 2). Interestingly, although TEM is not a quantitative technique (van der Pol et al., 2010), we found a greater number of exosomes per field in TEI-purified fractions and this number appeared slightly higher for TEX compared to VEX (Fig. 2). Furthermore, as described in the methods section, TEI-purified particles had to be diluted 1:10 before adsorption onto TEM grid to prevent vesicle overcrowding. Overall, our TEM analysis verified that both the procedures successfully isolated exosomes with an acceptable size range and morphology.

### 3.3. Analysis of exosomal miRNA content by qRT-PCR

As we surmised that there may be differences in the expression of chicken and MDV miRNAs in exosomes purified by different methods, we examined the expression of select miRNAs in exosome samples purified by each procedure (UC, n = 6; and TEI reagent, n = 6) by qRT-PCR. Both purification procedures permitted the detection of all analyzed miRNAs above the detection limit ($C_t < 35$) (Table 3). Based on the raw $C_t$ values, the miRNA threshold detection levels were on average 2.35 cycles lower in TEI reagent-purified exosomes, with an average of 0.96 and 3.73 cycles lower $C_t$ values observed in VEX and TEX, respectively. These data indicate that the total miRNA content in TEI reagent-purified exosomes is higher than that of UC-purified exosomes. The most and the least abundant miRNAs in VEX were similar between the purification procedures.

In contrast, the most and the least abundant miRNAs in TEX were quite different between the purification procedures. For instance, in TEX, gga-mir-27b-3p and -10b-5p were of highest and lowest abundance, respectively, in TEI-reagent purified exosomes, as opposed to gga-miR-2188-5p and -146b-5p in UC-purified exosomes (Table 3).

Likewise, in terms of miRNA expression in VEX, a strong correlation was observed between the two purification procedures (Fig. 3A, Pearson r = 0.85, $p < 0.0001$), whereas in TEX, weak correlation was observed between the two purification procedures (Fig. 3B, Pearson r = 0.55, $p < 0.0016$).

Finally, relative miRNA expression levels in VEX compared to TEX also exhibited a strong correlation between the two purification procedures (Fig. 3C, Pearson r = 0.9, $p < 0.003$). Expression of miRNA was uniquely dependent on the exosome purification procedure. However, in TEI-purified exosomes, MDV1-mir-M4, gga-mir-2188 and –mir-146b displayed 2.2-, 2.6- and 2.1-fold higher expression in VEX compared to TEX respectively (Fig. 3D). Similarly, gga-mir-99a and -21 displayed 2- and 4-fold greater level repression, respectively.

## 4. Discussion

In the present study, we compared the efficiency of two different exosome purification procedures, one based on serial ultracentrifugation steps and the other based on a polymer-based precipitation solution that is available commercially. Our starting sample was chicken serum obtained from an MD vaccine trial using commercial meat-type chickens that were either CVI988-vaccinated and protected against MD (as determined at necropsy) or unvaccinated and showed MD clinical signs, including visceral tumors. Particles purified by both the procedures were subject to quantitative comparisons in terms of the physical properties of the particles, the particle yield, and the miRNA content. Based on the precedent that cancer patient sera and transformed cell line supernatants harbor greater number of exosomes (Kalluri, 2016; King et al., 2012), we further refined our comparison to exosomes from the serum of vaccinated and protected chickens (VEX) and of unvaccinated tumor bearing chickens (TEX).

Using NTA and TEM imaging, we confirmed that both the procedures isolated particles within the size range of exosomes (30–150 nm) and with the spherical morphology. TEI reagent co-purified particles with greater size compared to UC and this size heterogeneity was higher for TEX particles. This result contrasted with a previous study, where exosome recovery efficiency of three commonly used commercial kits (ExoQuick, TEI, miRCURY) was compared to UC (Helwa et al., 2017). From pooled human sera, UC yielded particles with greater diameter compared to three commercial kits, although this result was not reproduced when the authors used individual human serum samples. In the same study, all three kits yielded higher number of particles compared to UC.

In another study, where the efficiency of exosome recovery from human serum was compared between UC and ExoQuick, a higher particle yield was demonstrated with ExoQuick (Caradec et al., 2014). In our study although the TEI reagent yielded slightly higher number of particles per mL, the difference was not significant. On the other hand, in our TEM field images, TEI-reagent purified fractions displayed consistently greater number of particles consistent with exosome size and morphology compared to UC-derived fractions. The source for this discrepancy is unknown.

Since NTA is an optical method that cannot distinguish protein aggregates from EVs, we surmise that UC-purified fractions may harbor a significant amount of non-exosomal aggregates of protein or lipoprotein (van der Pol et al., 2010). Supporting our conjecture, serum exosomes purified by UC on a 30% sucrose cushion contained

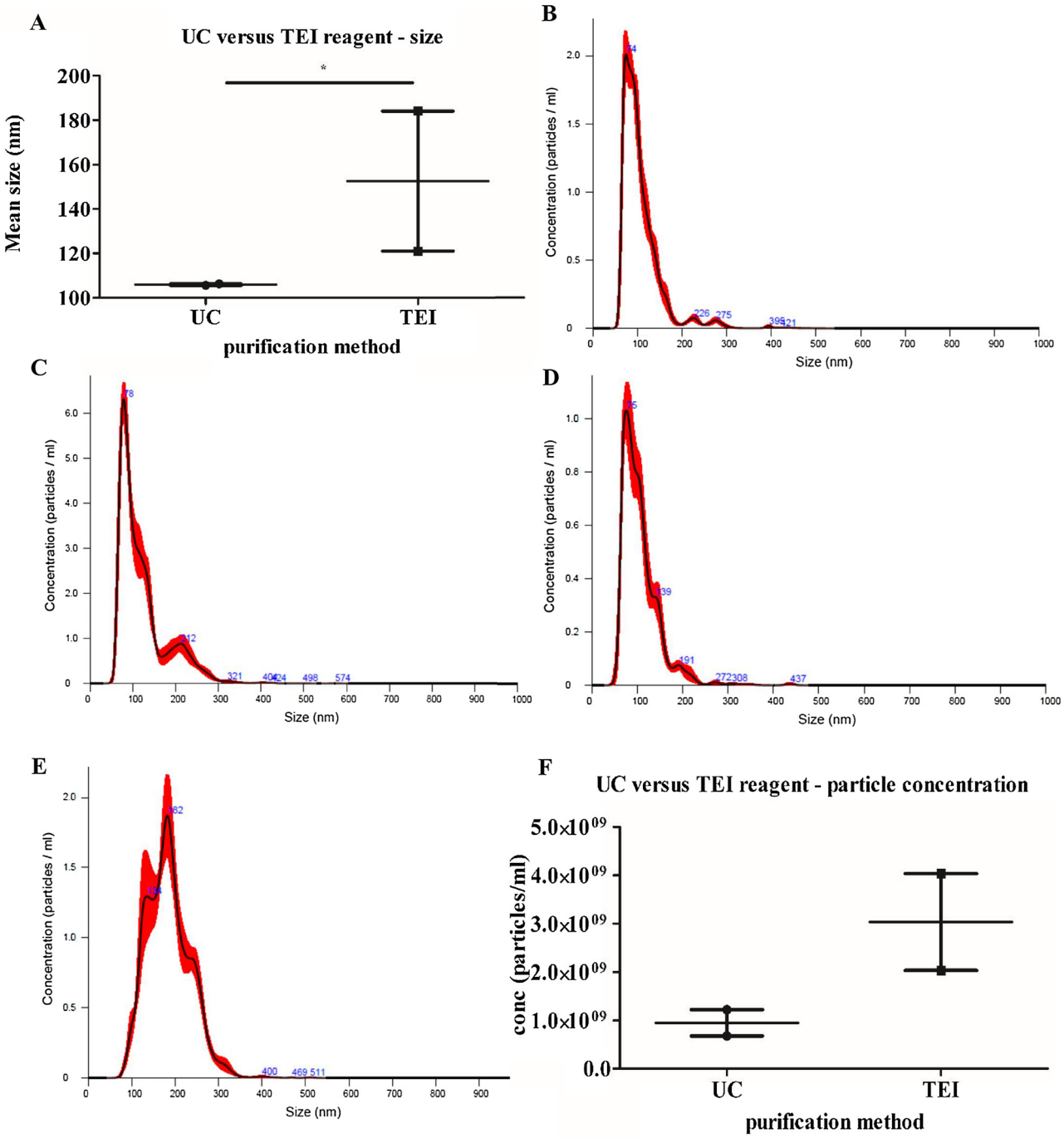


**Fig. 1.** Size and concentration profiles of particles purified by ultracentrifugation (UC) and Total Exosome Isolation precipitation (TEI) reagent. Panel A, shows the overall mean size (diameter) of particles yielded by UC and TEI reagent. Significant differences in particle sizes between the purification procedures was determined by Fisher's exact test. Error bars denote standard error of mean (SEM). Panels Panels B–C, show the size distribution and concentration profiles of vaccinate exosome (VEX) particles purified by UC (B) and TEI reagent (C). Panels D–E show the size distribution and concentration profiles of tumor exosome (TEX) particles purified by UC (D) and TEI reagent (E). Panel F, shows the overall particle concentration yielded by UC and TEI reagent. Error bars denote SEM.

significant amounts of albumin and IgG contaminants compared to those purified by ExoQuick™(Caradec et al., 2014). Additionally, viscous biofluids such as plasma (1.65 *centipoise* [Cp]) and serum (1.4Cp) were found to have lower sedimentation efficiency compared to less viscous cell culture conditioned media (1.1Cp) (Momen-Heravi et al., 2012). To overcome this issue, longer ultracentrifugation times were recommended, although ultracentrifugation for periods longer than 4 h can lead to vesicle rupture or fusion (Caradec et al., 2014; Cvjetkovic et al., 2014; Momen-Heravi et al., 2012).

An additional factor needed to be considered was the mode of action of polymeric precipitation reagents which capture and collect particles of a certain size range (60–150 nm) conforming to exosome size range in "polymer nets" that can be pelleted by a simple, low or high speed centrifugation using a bench top microcentrifuge (Peterson et al., 2015). Once pelleted, the supernatant containing excess polymer is discarded and the exosomes are resuspended in PBS to dilute any residual polymer and release the exosomes from polymer net. In this regard, polymeric reagents also co-purify protein complexes along with exosomes. Although the purity of particle fractions can be assessed by quantifying albumin and organelle marker proteins, such assessment is

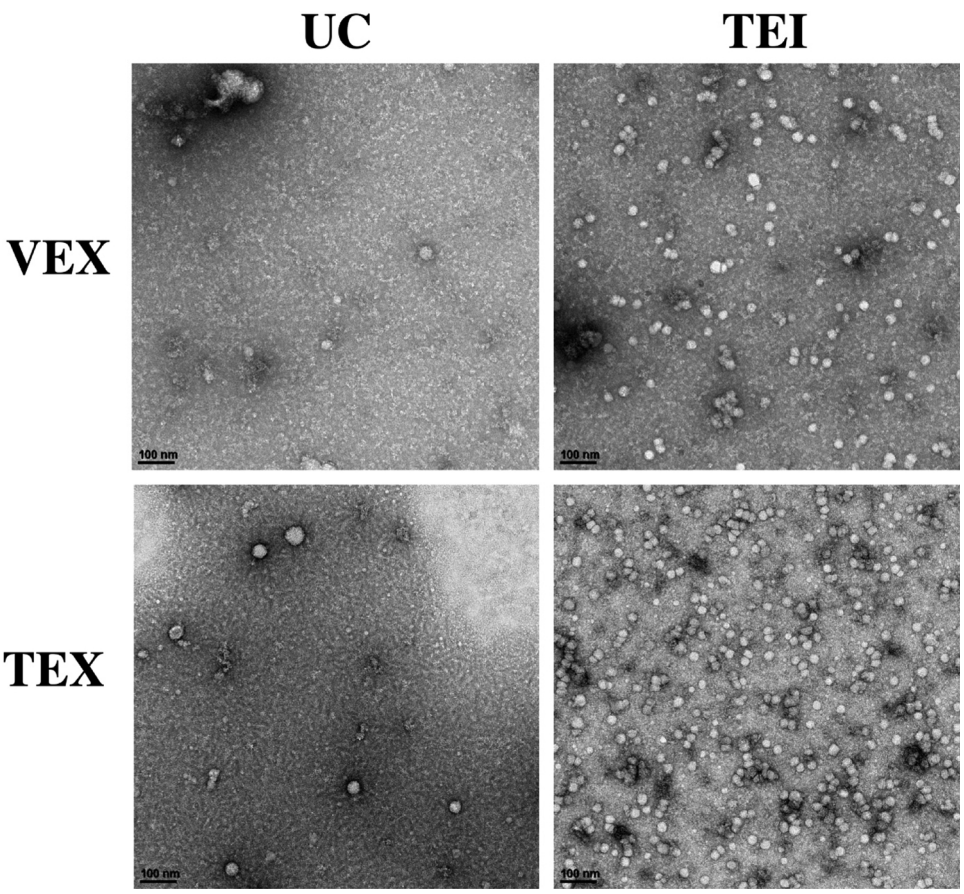


**Fig. 2.** Transmission electron microscopic (TEM) visualization of VEX and TEX purified using UC and TEI methods. The panels above show representative images of ~20 images per sample. The scale bar (100 nm) is shown at the lower left in each panel.

**Table 3**
Serum Exosomal miRNA $C_t$ values as Detected by qRT-PCR.

| | VEX | | TEX | |
|---|---|---|---|---|
| miRNA ID | UC | TEI | UC | TEI |
| gga-miR-2188-5p[b] | 26.5 | 26.4 | 26.1 | 24.7 |
| MDV1-mir-M4-5p[b] | 27.2 | 26.8 | 26.9 | 24.8 |
| MDV1-mir-M6-5p[b] | 27.5 | 27.8 | 27.3 | 25.2 |
| gga-mir-27b-3p[b] | 29.5 | 27.7 | 28.8 | 23.9 |
| gga-mir-146b-5p[b] | 30.2 | 30.1 | 30.2 | 28.4 |
| gga-mir-99a-5p[b] | 32.2 | 30.8 | 30.2 | 25.0 |
| MDV1-mir-M12-3p[b] | 32.3 | 30.0 | 31.5 | 25.9 |
| gga-mir-21-5p[b] | 33.0 | 30.9 | 31.5 | 24.6 |
| MDV1-mir-M8-5p[a,b] | 33.9 | 31.9 | 32.1 | 27.0 |
| gga-mir-10b-5p[b] | 34.9 | 32.6 | 33.0 | 28.2 |
| Average $C_t$ | 30.7 | 29.5 | 29.7 | 25.7 |

[a] miRNA level differences in VEX were statistically significant between the purification methods.
[b] miRNA level differences in TEX were statistically significant between the purification methods.

beyond the scope of current study.

In the present study, the miRNA expression profile in the serum exosome samples purified with the TEI reagent was compared to those purified using the standard UC method. Among the four MDV1 miRNAs profiled, two belonged to the oncogene *meq* cluster (MDV1-mir-M12 and –M4), whereas the other two belonged to latency-associated transcript (LAT) cluster (MDV1-mir-M8 and –M6) (Morgan et al., 2008). From our high throughput sequencing study of exosomal small RNAs in VEX and TEX from leghorn sera, we noticed a significantly higher expression of both *meq* and LAT cluster miRNAs in TEX relative to VEX (3.4, 10.8, 3.8 and 8.1-fold for -M12, -M4, -M8 and -M6 miRNAs, respectively). Upon qRT-PCR validation of the expression of above miRNAs, we confirmed the downregulation of –M12 and –M8 miRNAs at a comparable level in exosomes purified by both the procedures. Intriguingly, -M4 and –M6 miRNAs displayed higher expression in VEX relative to TEX. The reason for this discrepancy is unknown. Although –M12 and –M4 or -M6 and –M8 originate from the same primary transcript, differential post-transcriptional processing to yield mature miRNAs can lead to differential expression of the mature miRNAs. In addition, selective exosomal incorporation of miRNAs is an active process dependent on nucleotide (nt) motifs in miRNAs known as EXOmotifs that are recognized by SUMOylated hnRNPA2B1 (Villarroya-Beltri et al., 2013). In this regard, -M4 contained CCCU Exomotif that may allow its selective incorporation into exosomes irrespective of VEX or TEX, although –M6 lacked both CCCU and GGAG EXOmotifs.

MDV1-mir-M4 is a functional ortholog of cellular mir-155 and KSHV-mir-K12-11, and whose expression is correlated with the MDV1 virulence (Morgan et al., 2008). MDV1-mir-M4 is a known oncomiR that is nonessential for replication, but is highly-expressed in MD lymphomas or lymphoblastoid cell lines. Mutation of just 2 nts in the seed region of MDV1-mir-M4 completely abolished the transforming ability of the virus (Zhao et al., 2011). As instances of cellular miRNA expression contrasting with that of exosomes have been previously noted, and as hnRNPA2B1-dependent export of miRNAs in exosomes was proposed to provide a mechanism for eliminating undesired miRNAs (Valadi et al., 2007; Villarroya-Beltri et al., 2013), it is possible that MDV1-mir-M4 elimination in VEX may be a mechanism to prevent its cellular accumulation and thus its oncogenic functions.

Alternatively, MDV1-mir-M6 and –M8, although co-transcribed

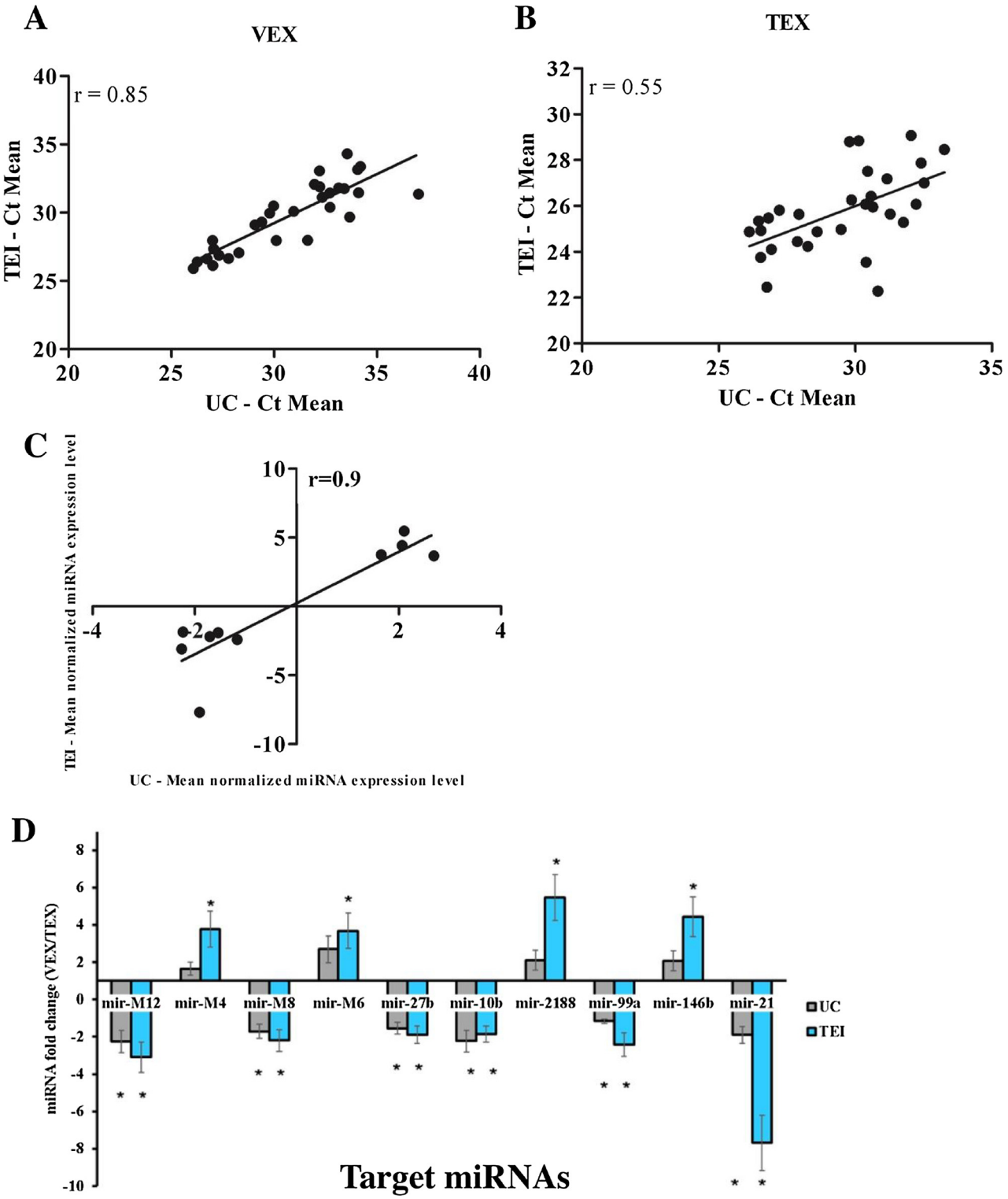


**Fig. 3.** Comparison of select miRNA expression levels between exosomes purified by UC and TEI procedures. Panels A–C show correlation analysis of miRNA expression levels in VEX (A), TEX (B) and VEX/TEX (C) between the exosome purification procedures. Pearson correlation coefficients (r) are shown for comparison. Relative MDV1 and *Gallus gallus* miRNA expression in VEX compared to TEX in UC-purified exosomes (grey bars), and TEI-purified exosomes (teal bars). Error bars denote SEM (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

from the same cluster, were previously shown to be differentially-expressed. MDV1-mir-M6 was demonstrated to be the least expressed or poorly processed in MDV1 (RB-1B) primary splenic tumors and the MSB1 lymphoblastoid cell line by deep sequencing, whereas a higher expression (3.8- and 8.5-fold for -5p and -3p, respectively) was noted in chicken embryo fibroblasts infected by the RB-1B strain of MDV (Burnside et al., 2006, 2008). MDV1-mir-M8 expression contrasted with that of –M6, where it displayed greater expression in RB-1B primary splenic tumors (3.5-fold) and the MSB1 lymphoblastoid cell line (23.5-fold) with basal expression in RB-1B-infected CEF (Burnside et al., 2006; Morgan et al., 2008). Exosomal MDV1-mir –M6 and –M8 expression levels in VEX compared to TEX also contrasted with each other

and reflected with those observed in RB-1B-infected CEF.

Similar to MDV1-mir-M4 and M6, qRT-PCR validation of chicken cellular miRNAs gga-mir-27b, -10b and -99a in VEX contrasted with the results from our high throughput sequencing study. The discrepant results obtained here might be attributed to the differences in our studies. The previous high throughput sequencing was performed on serum exosomes derived from leghorn sera whereas current qRT-PCR validation was performed on serum exosomes obtained from broiler sera. On the other hand, gga-mir-2188, -146b and -21 expression conformed to our high throughput sequencing results with a greater level of upregulation and repression (in VEX relative to TEX) seen in TEI-purified exosomes indicating higher exosome recovery by TEI reagent. With their *bona fide* roles as tumor suppressor miRNA and oncomiR respectively, gga-mir-146b and -21 expression correlated with good and poor prognosis in a wide variety of malignancies, thus can serve as biomarkers for vaccine-induced protection and MD positive status respectively (Correia et al., 2016; Ma et al., 2011).

Despite our findings, we are aware of our study limitations. First, our study selected only one of many available commercial exosome isolation reagent solutions from various sources; nonetheless we selected the most commonly used kit in the field. Second, our study only employed serum as a starting sample, and it is thus necessary to perform similar studies with other fluids such as plasma, ascites fluid and culture supernatants. Our ultimate intention, however, is to validate the most suitable technique for use in a routine diagnostic laboratory to identify or confirm serum biomarkers indicative of vaccine induced immune protection or systemic immune suppression. Third, we note that confirmation of the biological activity of purified exosomes is of prime importance prior to its translation for diagnostic use, although such confirmation assays are beyond the scope of this study. Fourth, we cannot exclude the lipoprotein or protein aggregate contamination of our purified fractions by both the procedures and the level of purity gained by each procedure will be assessed in our upcoming studies.

Taken together, our results demonstrate that commonly-used method to purify exosomes, UC, is relatively inefficient to recover exosomes from viscous fluid such as serum. While it may be an efficient procedure to purify exosomes from less viscous fluids such as culture media, urine and lavages, it is not the case for serum and also plasma. On the other hand, the use of TEI reagent to purify exosomes from serum is efficient, quick and can be performed with ease to obtain higher particle numbers for miRNA quantification.

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