Correspondence to: Inoshima, Y., Laboratory of Food and Environmental Hygiene, Department of Veterinary Medicine, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan

Tetsuya Yamada1, Yasuo Inoshima1,*, Tsukasa Matsuda2 and Naotaka Ishiguro1

ABSTRACT. Four methods were evaluated for isolating exosomes from bovine milk: (1) ExoQuick precipitation, (2) ultracentrifugation with ExoQuick precipitation, (3) ultracentrifugation with density gradient centrifugation, and (4) human milk exosome isolation. Methods 1 and 4 failed due to differences between bovine and human milk. Exosomes were efficiently isolated by ultracentrifugation with either ExoQuick precipitation (method 2) or density gradient centrifugation (method 3). The highest yield of exosomes was achieved using ultracentrifugation with ExoQuick precipitation, whereas higher quality exosome isolation with intact morphological structures was achieved by ultracentrifugation with density gradient centrifugation.

NOTE

Comparison of Methods for Isolating Exosomes from Bovine Milk

Tetsuya Yamada1, Yasuo Inoshima1,*, Tsukasa Matsuda2 and Naotaka Ishiguro1

1) Laboratory of Food and Environmental Hygiene, Department of Veterinary Medicine, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan
2) Graduate School of Bio-Agricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464–8601, Japan

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Exosomes, which are small membranous microvesicles (40–100 nm in diameter) containing microRNA, mRNA, and membrane and intracellular proteins, originate in endocytic compartments and are extracellularly released from a wide variety of mammalian cells [16]. In humans, exosomes are present in physiological fluids, including plasma [5], malignant effusions [3], urine [11], saliva, breast milk [9], and also bronchoalveolar lavage fluid [1].

Exosomes have been suggested to play a role in intercellular communication through either direct interaction of exosomal surface antigens with target cell receptors or via the transfer of RNAs and proteins from exosomes to target cells [6, 10]. Exosomes are also believed to be associated with the transmission of certain pathogens, including human immunodeficiency virus and Leishmania spp. [13, 17]. Interestingly, exosomes contain common RNAs, and membrane/intracellular proteins and cell-type and cell-specific proteins. Therefore, exosomes are considered to be possible novel biomarkers of diseases with particular interest in the diagnosis and prognosis of cancers [14].

Many biologically active molecules, such as cytokines and growth factors, are present in bovine milk. We determined that bovine milk exosomes contain mRNAs of major milk proteins and immune-related microRNAs [8]. More recently, Reinhardt et al. [12] identified 2107 proteins in bovine milk exosomes. It is conceivable that bovine milk exosomes also play important roles, such as immune system maturation and responsiveness, in the growth of infants. Bovine milk exosomes may also be useful as biomarkers for physiological and infectious diseases.

Optimization of isolation protocols for bovine milk exosomes has not yet been undertaken. The centrifugation with subsequent sucrose density gradient ultracentrifugation protocol used in our previous study required more than 24 hr to complete [8]. Recently, a precipitation solution called ExoQuick (System Biosciences, Mountain View, CA, U.S.A.) has become commercially available for the isolation of human exosomes from serum, ascites fluid, urine, and cell culture media [7, 15]. The advantages of ExoQuick are that it does not require a large sample volume or ultracentrifugation and that it is relatively rapid. The aim of this study is to evaluate four available methods for the isolation of bovine milk exosomes.

Bulk tank milk from healthy Holstein cows at the Gifu University Yanagido Farm was sampled and stored at 4°C until use on either the same or next day. Exosomes were isolated by each of 4 methods.

Method 1 (ExoQuick precipitation): Bovine milk samples were centrifuged at 3,000 × g for 15 min at 4°C using a Himac CF16RX centrifuge (T11A31 rotor; Hitachi Koki, Tokyo, Japan). Defatted milk (1 ml) was mixed with 1 ml of ExoQuick solution, and exosomes were precipitated according to manufacturer’s instructions. However, the resulting pellet could not be resuspended in phosphate-buffered saline (PBS) due to the precipitation of many proteins, such as casein. We concluded that ExoQuick alone was not sufficient for exosome separation in bovine milk, but that successive ultracentrifugation and filtration were also required in order to remove these proteins.

Method 2 (ultracentrifugation with ExoQuick precipitation): Bovine milk samples were centrifuged at 5,000 × g for 30 min at 4°C using a Himac CF16RX centrifuge (T11A31 rotor) to remove milk fat globules (MFGs), mammary gland-derived cells, and cell debris. Defatted milk was then subjected to three successive ultracentrifugations at 4°C in a Himac CP60E ultracentrifuge (P42A rotor; Hitachi Koki): 12,000 × g for 1 hr, 35,000 × g for 1 hr, and 70,000 × g for

3 hr. The supernatant was passed sequentially through 10.0-, 0.45-, and 0.22-µm filters (Millex; Millipore, Cork, Ireland), and 1 ml of the filtered supernatant (called whey or milk serum) was mixed with 1 ml of ExoQuick and incubated at 4°C for 12 hr. After centrifugation at 1,500 × g for 30 min at 4°C, the pellet was resuspended in 100 µl of PBS and the suspension was examined using a JEM1200EX (JEOL, Tokyo, Japan) or an H-7600 electron microscope (Hitachi, Tokyo, Japan). Isolated exosomes were not homogenous in size, being either 50 to 100 nm or 100 to 200 nm in diameter with rough surfaces (Fig. 1A). Unidentified structures were also observed, indicating that proteins isolated by method 2 contain both exosomes and other types of proteins or structures.

Method 3 (ultracentrifugation with density gradient centrifugation): We used the protocol from our previous study [8] with modifications. Whey from 100 ml of milk was ultracentrifuged at 100,000 × g for 1 hr at 4°C. The pellet was resuspended in 1 ml of PBS, and the suspension was layered on a linear sucrose density gradient (SDG) (10%–40%, w/v) solution (9 ml) and ultracentrifuged at 200,000 × g for 18 hr at 4°C in a Himac CP60E ultracentrifuge (P40ST swing rotor; Hitachi Koki). Sequential fractions (0.9 ml) were withdrawn from the top of the tube and numbered from 1 to 10. Western blot analysis confirmed the presence of exosomes with densities of 1.10 (fraction 7) to 1.19 g/ml (fraction 10) (data not shown). As the isolation from fraction 10 included unidentified structures observed by electron microscopy (data not shown), exosomes from SDG fractions 7 to 9 were diluted 10-fold in PBS, purified using an AmiconUltra centrifuge filter Ultrapac-30k (Millipore), ultracentrifuged at 100,000 × g for 1 hr at 4°C in an Optima MAX-XP ultracentrifuge (MLA-55 rotor; Beckman Coulter, Brea, CA, U.S.A.), and examined by electron microscopy. Isolated exosomes were homogenous in size, approximately 50 to 100 nm in diameter, and had smooth surfaces (Fig. 1B).

Method 4 (human milk exosome isolation): A protocol for isolating exosomes from human breast milk [2] was attempted for comparison purposes. However, unlike human milk, defatted milk could not be filtered, and this method failed at this step.

On Western blot analysis, exosome isolations from bovine milk using methods 2 and 3 showed bands at 50 and 100 kDa that reacted with anti-MFG-epidermal growth factor 8 (MFG-E8) [4] and anti-CD9 (ab3923; Abcam, Cambridge, UK) antibodies, respectively (Fig. 2). Exosomes were isolated in greater numbers by method 2 than by method 3 (Fig. 2). Further studies are required to elucidate the factors causing differences in morphological features between methods 2 and 3.

In this study, we showed that method 2 (ultracentrifugation with ExoQuick precipitation) is useful for rapid isolation and increased exosome recovery, whereas method 3 (ultracentrifugation with density gradient centrifugation) is suitable for efficient purification of exosomes with native morphology intact. Rapid and increased recovery in method 2 makes it possible to analyze the kinetics of mRNA or protein expression in exosomes. On the other hand, isolations of higher purity by method 3 can be used to examine the relationship of exosomal proteins to physiological or disease status of the host without any involvement or contamination of other free proteins in milk. The optimization of methods 2 and 3 will be useful for future analyses of biological and immunological functions of bovine milk exosomes.

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