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Determination of Bovine Proteins using Agarose Gel Electrophoresis Comparing lipoproteins to a Wet Chemistry Technique

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ABSTRACT

Serum lipoprotein quantification can tell you how well dairy cattle's hepatic and metabolic systems are working as well as how much lipid is being mobilized. Reagents designed for human lipoproteins are used in automated assays run on tabletop chemistry analyzers and with commercially available kits. The application of these assays for analyzing bovine lipoproteins may be complicated by the significant physical and chemical variations between those proteins and those found in humans and cattle. In order to semi-quantify the High-Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL) fractions by optical densitometry, 56 Holstein cows' serum lipoproteins were prospectively examined in this study. The electrophoretic lipoprotein separation pattern was verified by ultracentrifugation. The results of the electrophoretic approach were compared to estimated LDL cholesterol as well as those from direct measurements of HDL cholesterol, total cholesterol, and Triglyceride (TG) concentrations on a Roche chemical analyzer. It was unable to compute the correlation between these approaches for LDL and it was bad for HDL (Passing-Bablok regression line: y = 30.31 + 0.853x). In 25 of the 56 samples, automated HDL readings were equal to or greater than the total cholesterol level. 18 samples had TG amounts above the reference range, and on average, 96% of the cholesterol in these samples was determined to be HDL by an automated approach and 78% by electrophoresis. Our findings call into question the precision of the Roche automated assay in quantifying bovine lipoprotein fractions given that it is physiologically impossible to have more cholesterol within the HDL fraction than in the total serum fraction and the increased proportion of TG found in LDL and very-low-density lipoprotein.

KeyWords: ***Very-Low-Density Lipoprotein; Low-Density Lipoprotein (LDL);) EDTA***

A triglyceride (TG) and cholesterol ester core is encircled and stabilised by phospholipids, unesterified cholesterol, and proteins to form lipoproteins, which are macromolecular complexes. Chylomicrons, Very-Low-Density Lipoprotein (VLDL), intermediate-density lipoprotein, Low-Density Lipoprotein (LDL), and high-density lipoprotein are the four main kinds of lipoproteins in cows (HDL). Ultracentrifugation, the de facto method for quantifying lipoproteins, is time-consuming, labor-intensive, and unsuitable for routine diagnostic testing [1,2]. Reagents created for the quantification of human lipoproteins are used in automated tests run on tabletop chemistry analyzers and commercially available kits.

These tests typically use one of two fundamental approaches. The first technique involves measuring cholesterol repeatedly using enzymes both before and after the LDL lipoprotein particles are chemically precipitated. In the second approach, LDL, VLDL, and chylomicrons are selectively solubilized using detergents, polyanions, or both. Thus, only HDL remain structurally capable of reacting with the second assay step's enzymatic detection of cholesterol and cholesterol esters. The hydration density and chemical makeup of the various lipoprotein classes in cattle and people differ substantially. Lipids produced by the metabolism of the cow, presumedly ruminal hydrogenation, are substantially more saturated than those produced by human metabolism [3]. In comparison to human HDL, bovine HDL has a lower hydration density, higher protein content that is exposed to the surface, a higher percentage of phospholipids and cholesteryl esters, and an overall different lipid composition [4-7]. The majority of studies

examining the impact of diet, reproductive stage, and lactational status on HDL concentration in cattle do not provide primary or cited method validation data, despite the fact that there are known differences between the physical characteristics and composition of bovine HDL and human HDL.

In this study, we tested the hypothesis that wet chemistry techniques created for human samples would not be reliable for cow samples.

We compared the Roche Hitachi Modular P wet chemistry approach to an electrophoretic method in order to evaluate this theory. Because it physically separates bovine LDL and HDL in their natural condition and can be applied to several samples, electrophoresis was chosen as the comparison approach. The pattern of the bovine lipoproteins' electrophoretic separation was verified using density gradient ultracentrifugation. A cholesterol esterase/oxidase colorimetric test was used to assess the total cholesterol concentrations in serum samples submitted to the Animal Health Diagnostic Center (Ithaca, NY) for diagnostic purposes (Roche). 56 samples in total, all from mature Holstein cattle, were found. The biochemical examination of residual samples, including triglyceride concentration, a repeat of total cholesterol concentration, and measurement of HDL concentration using Roche's third generation HDL reagent on a Hitachi ModP analyzer, was done within 48 hours. The TG concentrations were measured using a reagent that includes a lipase enzyme to hydrolyze the bonds between the glycerol and fatty acid chains, coupled to enzymatic and colorimetric detection of the freed glycerol backbone (Roche). Agarose gel electrophoresis determination of bovine lipoproteins compared with a wet chemistry method Both total cholesterol and triglyceride assays were performed in accordance with the standard operating procedures of the Cornell Veterinary Clinical Pathology laboratory after daily quality control confirmation of assay performance. The LDL cholesterol concentration was calculated. Separate blood samples were collected for the isolation of lipoproteins by ultracentrifugation into EDTA tubes from cows at the Cornell University Teaching Dairy under Animal Care and Use Protocol 2007–0146. The majority of studies examining the impact of diet, reproductive stage, and lactational status on HDL concentration in cattle do not provide primary or cited method validation data, despite the fact that there are known differences between the physical characteristics and composition of bovine HDL and human HDL

Each serum sample (15 mL) was prepared by adding 5 mL of loading buffer (60 mM sodium-barbital buffer, 0.1% bromophenol blue in double-distilled water), followed by the volumetric loading of 15 mL into a 1% agarose gel (Agarose Unlimited) (Sigma-Aldrich). In 60 mM sodium-barbital buffer running buffer, lipoproteins were separated by horizontal electrophoresis at 80 V for 55 min (BioRad) (Sigma-Aldrich). Gels were stained with 0.18% Sudan black B (Sigma-Aldrich) in 70% ethanol for a whole night at room temperature (20-22°C). Gels were detained for 2 to 3 hours in a 15% acetic acid/20% acetone solution in double-distilled water, until the background was light grey to transparent and the bands were clearly visible. Lipoprotein bands were measured by converting the pixel intensity of the scanned lane into a linear peak plot and then using National Institutes of Health Image J software to calculate the area under the curve for each defined peak. Gels were scanned (Epson Perfection V500) as negative pictures (Figure 1).

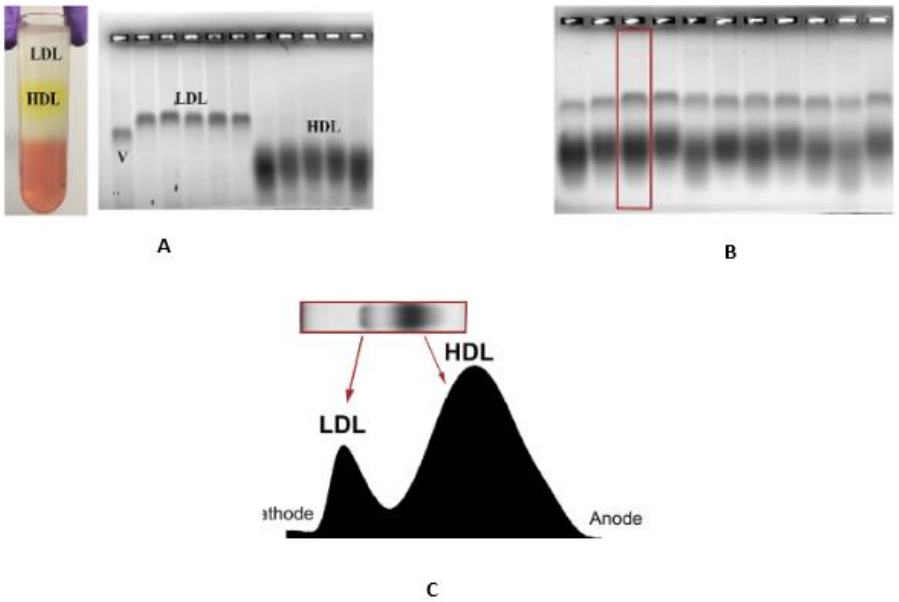

Fig.1 **An illustration of a densitometry plot and lipoprotein fractions separated by ultracentrifugation that were subjected to**

agarose electrophoresis to validate the migration patterns in the gel. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) separation in ultracentrifuged bovine plasma, as well as confirmation of the LDL and HDL movement patterns in agarose electrophoresis (A, left) (A, right). Due to the extremely low quantities of VLDL (V) in each individual cow, samples from 5 different bovine plasma samples were isolated by ultracentrifugation and electrophoresed after dialysis to remove extra salt. These samples are shown on the gel stained with Sudan black B.

Examples of electrophoresed and dyed lipoproteins from 16 cows are shown on an agarose gel (B). The related densitometry plot is shown below the gel, and it is used to determine the area under each peak's curve. This area under the curve is then converted to a percentage of total lipoprotein (C).

All of the substances needed for ultracentrifugation were bought from Sigma-Aldrich. The following stock density gradient solutions were created: In 1 L of water containing 1% (wt/vol) disodium EDTA, 1.006 g/dL equals 8.766 g of sodium chloride, while 1.346 g/dL equals 153 g of sodium chloride and 354 g of potassium bromide. Df = (d1 v1) + (d2 v2)/v1 + v2, where Df is the final density, d1 and d2 are component densities, and v1 and v2 are the corresponding volumes, was used to combine these stock gradient solutions. In order to prepare the plasma for ultracentrifugation, 13 mL of EDTA-anticoagulated plasma were first diluted to a density of 1.34 g/dL using solid KBr, then 4 mL of d = 1.18 g/dL solution, 6 mL of d = 1.091 g/dL solution, 6 mL of d = 1.063 g/dL KBr, NaCl EDTA, and The gradient was centrifuged in a SW 32Ti swinging bucket rotor at 126,000 g for 22 hours at 4°C with gradual acceleration and no brake applied. Sequential aspiration was used to separate the fractions, starting at the tube's top: VLDL = 2 mL, LDL = 4 mL, HDL = 8 mL (11–18 mL), and the LDL/HDL interface was removed.

The samples' median total cholesterol level was 2.77 mmol/L (107 mg/dL), with values ranging from 1.4 to 6.66 mmol/L (54 to 257 mg/dL). Total cholesterol levels ranged from less than 2.59 mmol/L (100 mg/dL) in 16 samples to more than 3.88 mmol/L (150 mg/dL) in 28 samples, and more than 3.88 mmol/L (150 mg/dL) in 12 samples. Hemolysis-positive samples were disqualified. It has been previously reported that the manual electrophoresis method is precise, with intraassay coefficients of variation of LDL and HDL (n = 40 repetitions) being 11.4 and 2.27%, respectively [8]. The Roche assay's manufacturer-provided coefficients of variance (n = 21 replicates) for the low- and high-level human serum controls are 1.3% and 1.2%, respectively. Poor HDL correlation was found between the two approaches (Passing-Bablok regression line: y = 42.3 + 0.68x). LDL could not be determined because automated HDL readings in 25 of the 56 samples were equal to or higher than the total cholesterol concentration. The use of linear regression analysis is demonstrated to enable data visualisation (Figure 2). LDL was identified by electrophoresis in all samples (average of 18% of total lipoproteins, SD = 0.8%, n = 56). The TG values in 18 samples were higher than the reference interval, and in these samples, the automated approach detected an average of 96% of the cholesterol as HDL while the electrophoresis method detected an average of 78% of the lipoprotein content as HDL.

According to electrophoresis results, there was a modest correlation between triglyceride concentrations and LDL cholesterol levels (r = 0.39).

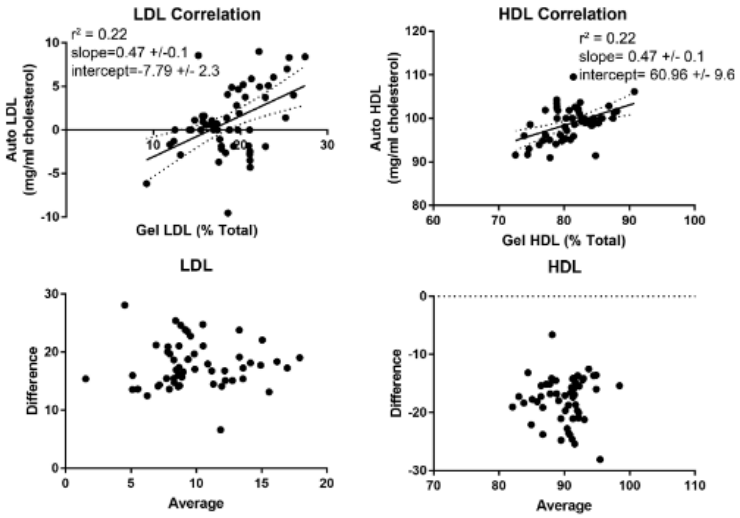

Fig. 2 **Low-density lipoprotein (LDL; right) and high-density lipoprotein (HDL; left) levels were compared using linear regression (top row of panels) and Bland-Altman plots (bottom row of panels) utilising the automated wet chemistry method (Auto) and agarose electrophoresis (Gel).**

To accurately assess the metabolic condition and health of both individual animals and herds, dairy cattle must have accurate measurements of their lipid metabolism. Since correlations might conceal biases, using correlational comparisons to validate tests is insufficient. It is necessary to understand the accuracy and biases involved in each approach employed before comparing results from different studies. Therefore, when a technique or assay created for use on human samples is used in veterinary research, comprehensive validation studies including Bland-Altman comparisons must be performed [9]. In this investigation, we showed that when used to measure bovine HDL concentrations, the Roche HDL3 reagent performed poorly in comparison to electrophoresis. Numerous times, results that were physiologically impossible to produce were produced. The proportional proportions of LDL and HDL that we assessed were comparable to those found in other research that employed comparable electrophoretic methodologies [10,11]. Our results raise concerns about the precision of the Roche automated assay in measuring bovine lipoprotein fractions, and similar concerns might apply to other wet chemistry techniques as well.

It is not unexpected that Roche reagents designed to react with human HDL produce ambiguous results in this species considering the distinct chemical characteristics of bovine lipoproteins. Our electropho- retic results agree more with research using Technicon RA 1000 from Bayer [7]. It's possible that the Bayer reagents work well with bovine lipoproteins. Studies would be necessary to support or disprove this theory.

The relative absence of severe values in the current study is one of its limitations. Dairy cattle's biology, which consistently tends to be an HDL-rich animal with a very little level of LDL, plays a role in this. Poor HDL correlation was found between the two approaches (Passing-Bablok regression line: $y = 42.3 + 0.68x$). LDL could not be determined because automated HDL readings in 25 of the 56 samples were equal to or higher than the total cholesterol concentration. The use of linear regression analysis is demonstrated to enable data visualisation (Figure 2). LDL was identified by electrophoresis in all samples (average of 18% of total lipoproteins, SD = 0.8%, n = 56). The TG values in 18 samples were higher than the reference interval, and in these samples, the automated approach detected an average of 96% of the cholesterol as HDL while the electrophoresis method detected an average of 78% of the lipoprotein content as HDL. These tests can therefore be applied across a wide range of species barriers.

While potentially requiring reagents that are species-specific, electrophoretic techniques and other physical methods of lipoprotein separation are typically more time-consuming and expensive. Studies that compare certain animal species or between different disease states while using a consistent technique are less vulnerable to the confounding effects of assay inaccuracy than those that aim to determine a lipoprotein concentration threshold that is useful for diagnosis. When planning and carrying out a study, it is important to keep as-say constraints and the necessity for cross-species validation in mind.

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