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SPERMOVA

Spermova 2022; 12(2): 197-202

Artículo original – original article

DOI. 10.18548/aspe/0010.26

PROTEOMIC APPROACHES OF SEMINAL PLASMA TO EVALUATE THE SEMINAL QUALITY OF BULLS RAISED IN THE COLOMBIAN TROPICS

Analisis del proteoma del plasma seminal para evaluar la calidad seminal de toros criados en el trópico Colombiano

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Recibido: 09/11/2022

Aceptado: 15/03/2023

Publicado: 31/03/2023

ABSTRACT

The role of seminal plasma proteins during cattle fertilization is well described. However, many of their biological functions are still unclear. Based on previous research, the idea that differences in proteomic profiles of seminal plasma could explain the fertility differences in bulls of diverse breeds was hypothesized. Two-dimensional electrophoresis was used to detect the differences in seminal plasma protein profiles of Sanmartinero (SM) and Zebu Brahman (ZB) bulls in tropical conditions of Colombia and to establish a relationship between these profiles and seminal quality parameters. Semen was collected from 10 bulls of each breed, and quality parameters, including sperm concentration, motility, and cell viability, were determined. Seminal plasma was used to perform two-dimensional electrophoresis. The statistical analysis employed t-tests and Pearson's correlation tests. Results showed that sperm concentration and viability showed higher values in SM semen ($P<0.05$ and $P<0.001$, respectively). A comparison of two-dimensional electrophoretic profiles from both breeds showed differences in the relative amount of 10 spots, five were higher in SM, and the other five were higher in ZB profiles. Additionally, we found a correlation between the quantity of the spot identified as an acid seminal fluid protein (aSFP) from SM and sperm viability ($r=0.64$, $P<0.001$). The findings suggested a tight relationship between seminal plasma proteins and sperm quality, enlarging the possibility of using proteomic tools to enhance the biotechnology tools such as semen cryopreservation, in vitro fertilization (IVF), or embryo production.

Keywords: Proteomics; Reproduction; 2D electrophoresis; Seminal plasma; Sperm quality.

RESUMEN

El papel de las proteínas del plasma seminal durante la fecundación en el bovino está bien descrito. Sin embargo, muchas de sus funciones biológicas aún no están claras. Con base en investigaciones previas, se planteó la idea de que las diferencias en los perfiles proteómicos del plasma seminal podrían explicar las diferencias de fertilidad en toros de diversas razas. Se utilizó electroforesis bidimensional para detectar las diferencias en los perfiles de proteína plasmática seminal de toros Sanmartinero (SM) y Cebú Brahman (ZB) en condiciones tropicales de Colombia y establecer una relación entre estos perfiles y los parámetros de calidad seminal. Se recolectó semen de 10 toros de cada raza y se determinaron los parámetros de calidad, incluida la concentración de esperma, la motilidad y la viabilidad celular. Se utilizó plasma seminal para realizar electroforesis bidimensional. El análisis estadístico empleó pruebas t y pruebas de correlación de Pearson. Los resultados mostraron que la concentración y la viabilidad de los espermatozoides mostraron valores más altos en el semen SM ($P<0.05$ y $P<0.001$, respectivamente). Una comparación de los perfiles electroforéticos bidimensionales de ambas razas mostró diferencias en la cantidad relativa de 10 puntos, cinco fueron más altos en los perfiles SM y los otros cinco fueron más altos en los perfiles ZB. Además, encontramos una correlación entre la cantidad de la mancha identificada como una proteína de líquido seminal ácido (aSFP) de SM y la viabilidad de los espermatozoides ($r = 0,64$, $P <0,001$). Los hallazgos sugirieron una estrecha relación entre las proteínas del plasma seminal y la calidad del esperma, ampliando la posibilidad de utilizar herramientas proteómicas para mejorar las herramientas

biotecnológicas como la criopreservación de semen, la fecundacion in vitro (FIV) o la producción de embriones.
Palabras clave: Proteómica; Reproducción; electroforesis 2D; plasma seminal; calidad del esperma.

INTRODUCTION

The reproductive performance of bulls is directly related to the productive averages in the cattle industry (Rahman et al., 2017). Thus, enhancing sperm quality in bulls, or increasing their semen freezability, can improve the birth rates and herd productivity. Is widely known that seminal plasma contains a variety of proteins whose biological functions are related to seminal quality and fertility (Druart et al., 2019). Seminal plasma proteins confer to sperm cells the abilities mainly related to motility, cell membrane integrity, protection against reactive oxygen species (ROS), oviduct reservoir formation, sperm capacitation, acrosome reaction, and interaction with the pellucid zone (Pardede et al., 2020). In this sense, several studies have focused on establishing the relationship of these proteins with cattle fertility (Muhammad Aslam et al., 2018). For instance, acid Seminal Fluid Protein (aSFP) present in cauda epidydimal fluid and seminal plasma has been related to mitochondrial activity, sperm motility, lipid peroxidation (Bustamante-Filho et al., 2014; Rickard et al., 2015), and fertility in the Zebu Nelore breed, showing that the amount of aSFP is around 8.5 times higher in the sperm membranes of high fertility bulls than in those with low fertility (Roncoletta et al., 2006). Other proteins such as Clusterin, Tissue Inhibitor of Metalloproteinase 2 (TIMP-2), Osteopontin, and Z13 spermadhesines, have also been related to bovine male fertility (Muhammad Aslam et al., 2018; Ugur et al., 2022).

In this sense, since the molecular features of the seminal plasma of Colombian Creole bulls are not well known, this work is the first description of seminal plasma protein profiles from Colombian breeds with the purpose to drive further research toward the exploration of the current biodiversity in the country. This work aimed to explore the relationship between seminal plasma proteins and seminal quality parameters in two representative creole bovine breeds from the Colombian cattle industry, Colombian Sanmartinero (Bos taurus) and Zebu Brahman (Bos indicus), using electrophoretic profiles to characterize seminal plasma proteins.

MATERIAL AND METHODS

Semen collection and seminal quality parameters

Three semen samples per animal from 10 SM and 10 ZB bulls (3 to 4 years of age) were collected by electroejaculation. Semen collection and animal management procedures were approved by Agrosavia´s Bioethical Committee (2005 act SI-F-16). Seminal quality parameters such as sperm concentration, sperm viability, and motility, were evaluated. Sperm concentration was measured by spectrometry at 620 nm using 10 µL of semen samples previously diluted with 2.9 % sodium citrate (1:200), employing a portable Mini-Spermacue (Minitube, Verona WI, USA). Sperm viability was measured by counting sperm cells stained with Carboxy Fluorescein Diacetate (CFDA) and Propidium Iodide (Harrison and Vickers, 1990). For this purpose, 2 µL of semen was diluted 1:50 with 1X Phosphate-buffered saline (PBS), and diluted samples were incubated at 37°C with 5 µL of 0.1 mM CFDA and 5µL of 0.075 mM Propidium Iodide. Stained cells were fixed using 0.1 % formaldehyde and green cells (undamaged cell membrane) or red cells (damaged cell membrane) were counted in 200 sperm cells per optical field, using a fluorescent microscope Nikon Eclipse E600 (Nikon Instruments Inc, Melville, NY, USA) with a FITC filter. Finally, sperm motility was estimated employing microscope observation of 8 µL per semen sample at 40 x of magnification in several fields.

Seminal plasma separation and protein quantification

Semen samples were centrifuged twice at 10.000 x g for 15 min at 4°C. The supernatant was filtered using nitrocellulose 0.22 µm filters (EMD Millipore Corp, Billerica, MA, USA). Then, 5 µM phenylmethylsulphonyl fluoride (PMSF) (Sigma Aldrich, St. Louis, CO, USA) was added as a protease inhibitor, and the seminal plasma was stored at -20°C. Total protein amount in seminal plasma was established through the Bradford assay (Bradford, 1976) employing 1 mg/mL of Bovine Serine Albumin (BSA) as a standard.

Two-dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (2D-PAGE)

Seminal plasma samples were prepared employing 100 µg of seminal plasma proteins diluted with hydration buffer containing 8 M of urea, 4% CHAPS, 50 mM dithiothreitol (DTT), and 2% ampholytes (BioRad, Hercules, CA, USA). To separate the proteins by isoelectric point (pI), the mixture sample-buffer was spread on a plastic tray and covered with a pH-immobilized strip with a pH range between 3 to 10 (one strip per sample). Strips were passively hydrated overnight and then focused through a linear voltage increase until 250 volts/hour, followed by a rapid voltage increase to 4,000 volts/hour using the BioRad PROTEAN isoelectric-focusing (IEF) device. Focused strips were equilibrated by sequential washes with 2% DTT followed by 2.5 % Iodoacetamide. Afterward, strips containing the pI separated proteins were immediately fixed to 10-20 % polyacrylamide gels using 1% agarose containing 2 % bromophenol blue. Running was performed in a Mini-PROTEAN cell chamber (Bio-Rad) at 85 volts for 180 min. To calculate pI and Mw, a two-dimensional standard was used (BioRad, 2D Standard 161-0320). Gels were developed with a staining solution of 0.1 % Coomassie brilliant blue and all gel images were acquired using the Gel Doc XR (Bio-Rad). Then, all images were analyzed using the PD Quest software (Bio-Rad) to build a virtual map of each breed. Spot numbers were randomly assigned by the image analysis software.

Statistical analysis

The correlation between the relative amount of each spot and the seminal quality parameters was calculated using Pearson's correlation test. Relative amounts of spots and seminal quality parameters breed were compared employing a student's t-test. All statistical analyses were performed with the SAS software (SAS Institute, Cary, NC, USA).

Identification of spots (MALDI-TOF)

The low molecular weight spots were cut from the two-dimensional gels and extracted by tryptic digestion. The protein extracted was dialyzed against 50 mM of ammonium bicarbonate (NH_4HCO_3) and mixed with 2, 6 dihydroxiacetofetone (1:1). Then, 1 µL of the mix was deposited on top of a ground steel plate. Samples were

analyzed by a linear method using an UltrafleXtreme MALDI-TOF with ion acceleration of 25 kv (Bruker Daltonics, Bremen, Germany). Mass spectra values obtained were analyzed and compared using the MASCOT server and the NCBI database.

RESULTS

The comparison of seminal quality characteristics between SM and ZB showed that sperm viability in SM semen appeared to be approximately 15 percent more viable than in ZB semen ($P < 0.05$). Furthermore, the sperm concentration of SM seminal fluid was approximately 40 % higher compared to the one of ZB ($P < 0.001$, Figure 1). There were no significant differences in sperm motility values.

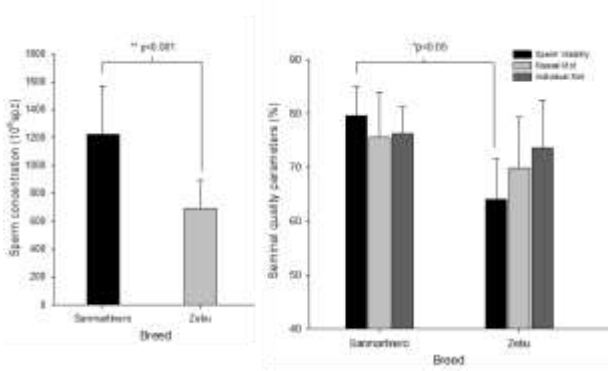

Fig 1. Comparison between seminal quality parameters of Sanmartinero and Zebu Brahman bulls. Significant differences are showed.

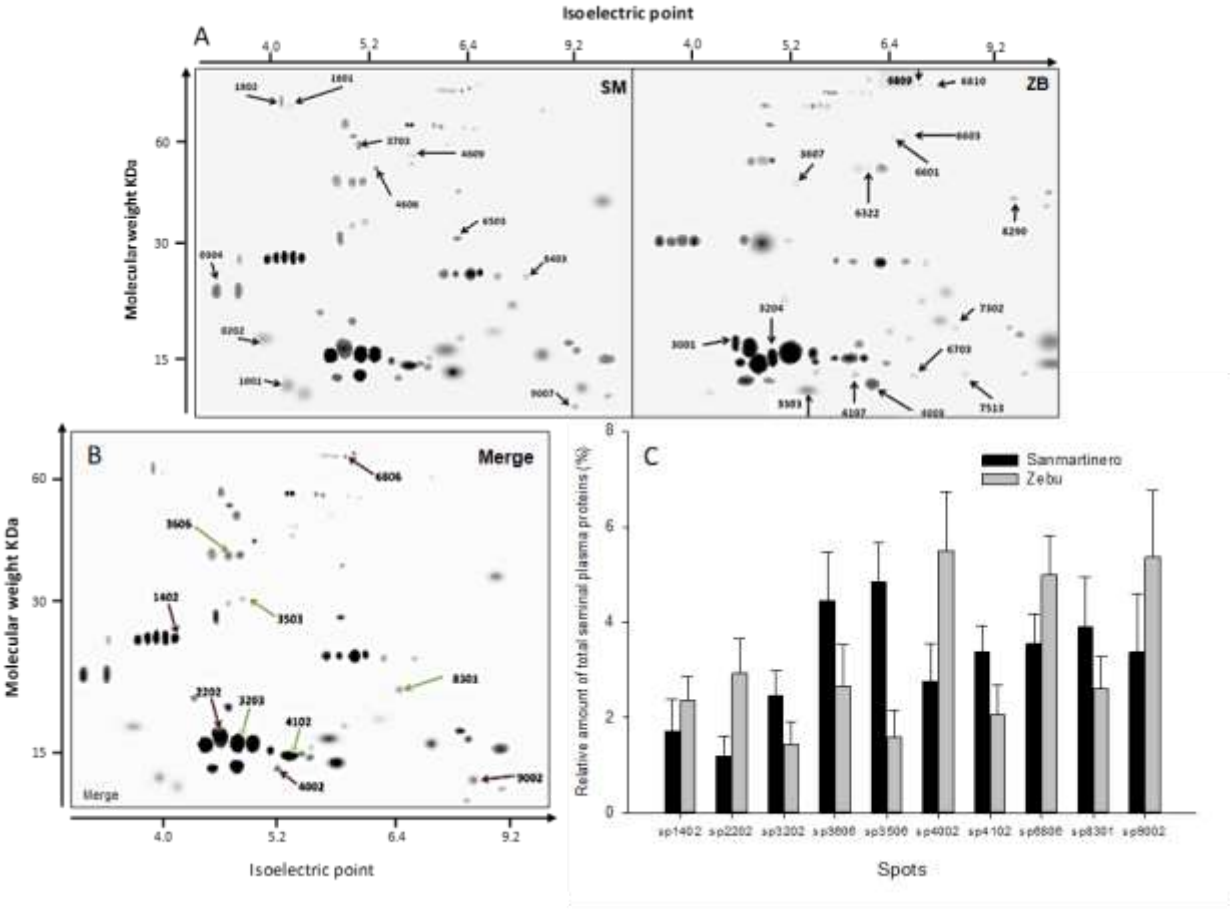

Fig 2. Electrophoretic profile differences between Sanmartinero (SM) and Zebu Brahman (ZB) seminal plasma. (A) Virtual master gels generated using the PD Quest software by matching 30 gels per breed. The arrows point to the spots that were unique for the maps of each breed. (B) Comparison of the relative amount of protein made by merging both virtual maps. Evidence showed significant differences in 10 spots as follows: the green arrows point to five spots with a high relative quantity in SM maps, and the black arrows point to five spots with a high amount in ZB maps. C) Comparison of the relative quantity of each spot with significant differences ($P<0.05$) between SM and ZB seminal plasma electrophoretic profiles.

The 2D-PAGE analysis of SM seminal plasma showed a mean value of 75 ± 12 protein spots, with MW ranging between 9.2 -78.5 kDa and pI values between 3.6 -9.8. Meanwhile, ZB profiles showed 67 ± 14 with MW values between 12.3 and 76.0 kDa and pI values between 3.9 and 9.8. The comparison of the virtual map images showed 11 protein spots that seem to be unique for SM profiles and 15 for ZB profiles (Figure 2A).

Since the biological functions of some proteins are highly concentration-dependent, the comparative analysis included the relative quantity of the spots in both profiles. The mean comparison test showed five protein spots with higher quantity in SM and five different protein spots higher in ZB samples (Figures 2B and 2C). Within the spots with different relative quantities, sp-3203, sp-2202, and sp-4002 were identified by MALDI-TOF as PDC 109 isoforms (BSP A1/A2) being the first one higher in SM profiles and the others higher in ZB profiles (Table 1).

Table 1. MALDI-TOF identifications for the spots in the BSP and Spermadhesin clusters with a different relative amount between SM and ZB profiles. The unspecific spot was identified after SEC separation in SM seminal plasma profiles.

Sp	Mw	pI	ID	Coverage (%)	MS/MS	Higher in maps of
2002	17.40	5.01	PDC 109	44	75	ZB
3203	16.20	5.10	PDC 109	44	72	SM
4102	15.30	5.73	Z13 Spermadhesin	55		SM
4002	15.17	5.68	PDC 109	82	102	ZB
4104	15.58	5.56	aSFP	74		No difference
Unsp.	15.66	5.70	Spermadhesin Z13 - BSP A1/A2 mixture		88	Only in SM profiles

Also, the statistical analysis showed a positive correlation (r=0.64, P<0.001) between the Sp-4104 spot (Mw 15.5 kDa; pI 5.6) and sperm viability in SM profiles (Figure 3A). On the other hand, sperm viability showed a statistically positive correlation (r=0.52, P<0.05) with the Sp-6701 spot (Mw 69.9 kDa; pI 5.5) in ZB samples.

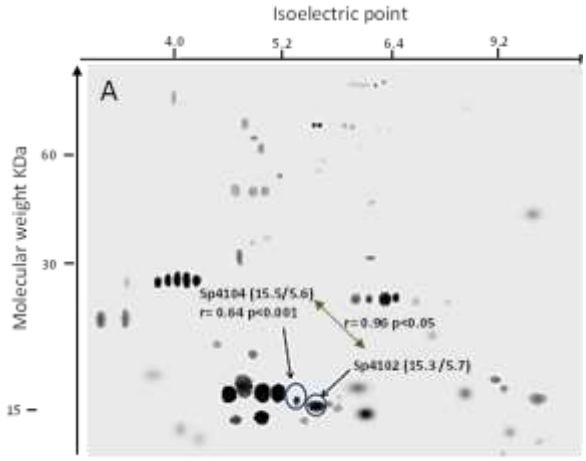

Fig 3. Spots analysis and identification in Sanmartinero (SM) seminal plasma. Master gel of seminal plasma proteins from SM; the arrows point to spots Sp-4104 and Sp-4102 whose relative amount were related to sperm viability percentages.

Additionally, other less strong correlations between the relative protein quantity in spots and sperm quality parameters were found, mostly in low molecular weight spots. Interestingly, some spots were even related to two seminal parameters. The relative protein quantities of Sp-3101 (Mw 13.5; pI 5.17) and Sp-9202 (Mw 17.24; pI 9.02) showed positive correlations with sperm motility and sperm concentration in SM semen samples. Furthermore, a correlation between the relative quantities of two different spots from SM seminal plasma was also found. Sp-4104 (Mw 15.58 kDa; pI 5.56) showed a high correlation (r=0.96 P<0.05) with Sp-4102 (Mw 15.3 kDa; pI 5.7).

DISCUSSION

Seminal quality parameters are relevant for an initial estimation of the male fertility status and competence. In this work, semen from SM bulls showed higher sperm viability and sperm concentration average values than ZB bulls. Since there are no previous references of fertility averages in SM bulls and ZB bulls, the comparison of their seminal quality parameters is the current best approach. Sperm viability percentages could point out to better reproductive behavior of SM bulls compared to ZB, and it could be explained from an adaptation perspective, suggesting that SM, as a Creole breed, would be better adapted to the typical environmental conditions and management of the Colombian tropics. However, we should develop more studies to extend knowledge regarding such suggestions.

The relationship between seminal quality and proteomics profiles was evaluated through a statistical correlation test. Using this approach, the relative protein quantity of Sp-4104, i.e. a low molecular weight spot, was found to be related to the membrane integrity of SM sperm cells. This spot was identified as an acidic Seminal Fluid Protein (aSFP), also known as Spermadhesin Z13, a 13 kDa peptide that belongs to the spermadhesines family, whose amino acid sequence contains four cysteines forming two disulfide bonds (Jobim et al., 2004; Pini et al., 2016). Reports have described three different isoforms of aSFP isolated from bulls' seminal plasma. These isoforms have different oxidation states of cysteine residues, which explains their protection capability in oxidative environments (Montanholi et al., 2016). Hence, even though aSFP does not have a role during fecundation, its protective effect seems relevant for sperm viability.

Although the correlation between sperm viability and the relative protein quantity of Sp4104 was found only in SM electrophoretic maps, aSFP had been previously reported in membrane fractions of sperm cells from Zebu Nelore animals. Interestingly, its relative quantity was described as 8.5-fold greater in bulls with highly efficient semen in artificial insemination procedures (Magalhães et al., 2016; Roncoletta et al., 2006). In addition, another electrophoretic characterization of seminal plasma from 16 Bos taurus samples

evidenced that a 12 kDa spot (pI 4.8), identified as aSFP, was more frequently found in the semen of bulls with "high freezing rates" (Gomes et al., 2020). In this sense, diverse works have evidenced a marked relation between aSFP and sperm freezability, since this protein participates in both sperm motility, through mitochondrial activity regulation (Bustamante-Filho et al., 2014), as well as the protection against lipid peroxidation; this last may be mainly due to the presence of disulfide bonds with redox capability.

On the other hand, the relative amount of Sp-4102, identified as Spermadhesin Z13, was tightly correlated with the relative quantity of aSFP in SM seminal plasma, which could be due to aSPF is, in fact, a Spermadhesin precursor. In this regard, previous works have discussed the presence of a Spermadhesin regulation system in the seminal plasma. This mechanism forms protein complexes that could work at the cellular level with influence sperm viability (Westfalewicz et al., 2021). Furthermore, the addition of purified spermadhesines can inhibit the adhesion of spermatozoa to the oocyte pellucid zone, and their affinity to heparin and galactoses suggest an active role in sperm capacitation. Interestingly, the relative amount of Spermadhesin Z13 from the seminal plasma of SM also showed a negative correlation with sperm motility ($r=-0.54$; $P<0.05$). These findings could be related to previous observations of seminal plasma from Holstein bulls, where high Spermadhesine Z13 levels have been linked to low fertility averages of cryopreserved semen, reducing the success of artificial insemination (Kumar et al., 2012). Another report from these authors concluded that heparin-binding spermadhesines belong to a protein complex related to sperm motility regulation, like BSP proteins (Perumal et al., 2016; Srivastava et al., 2012).

In the case of ZB bulls, a 28 kDa spot was related to sperm viability. Dalton et al. (2012) found a heparin-binding protein with similar features (around 31 KDa) identified as a fertility-associated antigen (FAA) in seminal plasma from Santa Gertrudis and Santa Cruz bulls (both with Brahman as a remote ancestor). This protein has been strongly associated with high fertility averages in bulls (Dalton et al., 2012). However, the attempt to find a relationship between FAA and fertility of other Zebu bulls, such as Nelore, has been unsuccessful suggesting that a unique molecule cannot be used as a fertility marker for bulls from different breeds and that these fertility markers could be breed-depend. More recently, FAA have been used to increase the post-thaw semen quality (Suprayogi et al., 2020).

Thus, the analysis of seminal plasma electrophoretic profiles offers an interesting overview that allows for detecting differences among breeds with different seminal quality parameters, as well as establishing the relationship between the protein content and the fertility potential. In this sense, proteomic profiles of seminal plasma could be used to select specific proteins as fertility markers for each breed under specific management conditions, and further, use these markers for the disposal or sire selection in cattle production systems. In this sense, the determination of protein profiles could become a key tool to explain observed differences in the reproductive performance of bulls from different breeds under equal conditions. In this way, the higher semen quality values observed in SM semen could drive future research towards experiments involving direct measurement of fertility or in vitro fertilization (IVF) trials, in which the molecular background should be strongly considered.

Conflict of interest

Los autores del presente artículo declaran no tener ningún conflicto de interés personal o económico con otras personas u organizaciones que puedan influir indebidamente con el presente manuscrito.

Author´s contribution

Conception and design: FR, JC; Experimental development: FR, RH, EN, MP; Data Analysis: FR, JGV, JC; Writing and editing: FR, JGV, JC; Drafting: FR, RH, EN, MP, JGV, JC; Critical revision of the article: FR, JC.

Acknowledgments

This work was developed within the framework of the Colombian Corporation for Agricultural Research (AGROSAVIA) and supported by Ministerio de Agricultura y Desarrollo Rural (MADR).

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