Dermatology Practical & Conceptual

Supplementary file

File S1:

Laboratory tests: We assessed 45 sera from patients with El Bagre EPF and 45 normal sera from the endemic area. The patients and controls were paired by demographics, including age, sex, nutritional status, and occupation. A human quality assurance review board approved the studies at the ESE Nuestra Senora del Carmen in Antioquia state, Colombia. The study participants signed informed consent forms, and sera was taken to be evaluated by indirect immunofluorescence (IIF). Additionally confocal microscopy (CFM) examinations were performed as previously described [2,8]. Only patients who fulfilled full diagnostic standards for El Bagre-EPF were included, as follows: i) patients displaying clinical and epidemiological features designated for this disease; ii) patients living in the endemic area; iii) patient autoantibodies presenting intercellular staining (ICS) between epidermal keratinocytes using antihuman IgG, IgM, Kappa, lambda, IgG4, Complement/C3, fibrinogen, and albumin using direct immunofluorescence studies (DIF) as well as IIF. Additional staining could be seen, including epidermal pericytoplasmic and/or dotted patterns between epidermal keratinocytes utilizing anti-human IgD, fibrinogen, and albumin antibodies. Positive staining with the antibodies was also defined as a dotted pattern along the dermal/epidermal basement membrane zone (BMZ) by either DIF or IIF as previously reported [2,8]. Likewise, positive staining could be seen in the lower part of the BMZ with the antibodies. An added polyclonal immune response could be seen in the epidermal corneal layer as well as in dermal appendageal structures as dotted ICS. Positive staining could be seen at dermal mesenchymal-endothelial cell junctions. Additional diagnostic criteria of El Bagre-EPF disease included the patient serum being positive by immunoblotting (IB) reactivity against desmoglein (Dsg) 1 as well as for envoplakin, periplakin, and BP230 plakin molecules as previously described [2,8]; v) the patient serum immunoprecipitated a Concanavalin A affinity-purified antigen, the bovine tryptic 45-kDa ectodomain portion of Dsg1; vi) the patient serum generated a positive result using an ELISA when screening for autoantibodies to El Bagre-EPF antigens and colocalization of patient autoantibodies with desmoplakins I-II, p0071, ARVCF, and MIZAP.

Indirect immunofluorescence (IIF): Our studies were performed as previously described [4–6]. Bovine tissue including intervertebral and vertebral tissue from 2– 4-year-old cattle were acquired from the abattoir within two hours of slaughter. Tangential sections were cut successively, in the plane tangential to the lamellae, from the outermost annular layer to the beginning of the nucleus pulposus pulpous (NP). Serial slices were taken at intervals of two slices (4 µm thick) as antigen sources for the IIF. These were incubated for four minutes (min) with 1X PBS and 3.5% paraformaldehyde (partial fixation). The slides were then washed twice with PBS for 10 minutes per wash, then moderately permeabilized, using 1X PBS with 0.1% Triton X-100 and 1% normal goat serum (for blocking and permeabilization) for 10 minutes under rotation, and rewashed again twice with PBS. Slides were then counterstained with 4′,6-diamidino-2 -phenylindole (DAPI) (Pierce, Rockford, Illinois, USA). For colocalization, we used commercial antibodies produced against DP-I–II (mouse monoclonal multi-epitope cocktail, Progen Cat. no. 65146) dilution 1:50, ARVCF polyclonal antibody (source guinea pig, tested in human and bovine) Cat no. GP155 (dilution 1:50); for its secondary, we used Alexa Fluor®555 goat-anti-guinea pig from Molecular Probes/Life Technologies/Thermo Fisher Scientific (Waltham, Massachusetts, USA). We also used plakophilin-4 (dil 1:50), Cat. no.651166, and a mouse monoclonal antibody for Myozap, MIZAP Cat no.651169. As a secondary antibody against DP-I–II, p0071, and Myozap, we used Texas red-conjugated goat anti-mouse IgG from Thermo Fisher. The antibodies against DP-I-II, ARVCF, p0071, and MIZAP were obtained from Progen Biotechnik in Germany. The samples were consistently run with positive and negative controls. We also used monoclonal *antibodies* specific for glial fibrillary acidic protein (*GFA*) conjugated with Alexa Fluor 594® from Thermo Fishers (Waltham, Massachusetts, United States). Two independent researchers with inter-rater reliability assessed the value of the experiments.

Confocal microscopy studies (CFM): These were performed as previously described [2,8]. Standard 20x, 40x and 100x objective lenses were used. Each frame included an area of 440 to 330 mm. Images were taken using an EZ-1 image analysis software (Nikon, Tokyo, Japan). For

colocalization experiments with the serum autoantibodies, we used the antibodies against DPI-II, ARVCF, p0071, and MIZAP.

Reflectance confocal microscopy (RCM): RCM images were acquired by means of near-infrared reflectance confocal laser scanning microscopes (Vivascope 1000s and Vivascope 1500s, Lucid Inc., Henrietta, New York), which use an 830 nm laser beam with a power of 35 mW. Each image corresponded to a horizontal section at a selected depth with an approximately 0.5 to 0.5 mm field of view, a lateral resolution of 1.0 mm and an axial resolution of 3–5 mm. A sequence of montage images ('block' images) was acquired for each lesion at the level of the IVD.

Statistical analysis: We used Fisher's exact test to assess two nominal variables (e.g., positive, and negative) of the antibody response. We recorded these data points: i) positivity of the El Bagre-EPF autoantibodies between patient cases and controls; ii) patient antibody results versus the commercial antibodies against Myozap, p0071, DP-I–II, and ARVCF. $P<0.05$ with a 95% confidence interval or more reflected statistical significance. We used the GraphPad QuickCalcs software from GraphPad Software (La Jolla, California, USA).