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Pathological Prion Protein in the Tongues of Sheep Infected with Naturally Occurring Scrapie

Cristina Casalone, Cristiano Corona, Maria Ines Crescio, Francesca Martucci, Maria Mazza, Giuseppe Ru, Elena Bozzetta, Pier Luigi Acutis, and Maria Caramelli*

CEA, Italian Reference Laboratory for TSE, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy

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Tongue involvement by prion spreading was shown to be a common outcome after oral or intracranial experimental challenge with scrapie and transmissible mink encephalopathy sources in rodent models. It is also known that bovine spongiform encephalopathy, which is pathogenic for humans, is experimentally transmissible to sheep and can lead to a disease indistinguishable from scrapie. A recent European Food Safety Authority opinion recommended research into PrP^{sc} accumulation in the tongues of ruminants. We report on the detection of PrP^{sc} in the tongues of seven scrapie-infected sheep by immunohistochemistry and Western blotting.

Recent studies on rodent models (1, 8, 10), have reported the detection of PrP^{sc} in tongue tissue after oral or intracranial experimental challenge with scrapie and transmissible mink encephalopathy sources. Unlike bovine spongiform encephalopathy, scrapie is not considered a risk to humans health (2), but it has been demonstrated that under experimental conditions sheep are easily infected by the bovine spongiform encephalopathy agent and that they carry abundant amounts of infectivity throughout most body tissues (6). A European Food Safety Authority opinion (3) recommends testing for PrP^{sc} presence and accumulation in ruminant tongues in order to facilitate risk quantification and assessment. In this study, we report on the presence of PrP^{sc} in the tongues of sheep infected with naturally occurring scrapie.

We studied 10 negative, regularly slaughtered adult sheep and 10 adult sheep positive by rapid test (Prionics-Check Western) coming from two different affected flocks in Piedmont and Tuscany, respectively (flock A, eight sheep of the Biellese breed, aged 20 months to 7 years; flock B, two sheep of the Sarda breed, aged 20 months and 2.5 years). The disease was confirmed by histology, immunohistochemistry (IHC), and Western blotting (WB) in the brainstem. Both sheep from flock B and four from flock A showed clinical signs of scrapie (tremors, emaciation, falling). No relevant differences in the amount of PrP^{sc} in the obex were found between animals with or without clinical signs. All animals had an ARQ/ARQ *PrP* genotype.

The tongue of each animal was cut into two halves, of which one was fixed in buffered formalin and the other was frozen. Two specular areas of each half were examined independently: one at the level of the apex and one from the corpus linguae. For IHC, 10 serial tissue sections, 5 to 6 μm thick, were obtained and numbered: sections numbered 1, 4, and 7 were stained to detect PrP^{sc} immunoreactivity; sections numbered 2, 5, and 8 and 3, 6, and 9, respectively, were stained using PGP 9.5 and neurofilament to investigate the possible involvement of cells and nerve fibers. All tissue sections were dewaxed and rehydrated by routine methods and then subjected to an antigen retrieval procedure (5). The sections were immersed in 98% formic acid for 20 min, washed in distilled water, and then autoclaved for 20 min at 121°C in distilled water. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 20 min at room temperature. To remove nonspecific tissue antigens, the sections were incubated with 5% blocking serum for 20 min at room temperature. Tissues were incubated overnight at 4°C with the primary antibody. PrP immunostaining was carried out with monoclonal antibody (MAb) F99/97.6.1 (9) (1:1,000 dilution; VMRD Inc., Pullman, WA) or MAb L42 (4) (1:250 dilution; R-Biopharm, Darmstadt, Germany). Anti-PGP 9.5 polyclonal antibody (1:100 dilution; Dakocytomation, Carpinteria, CA) and anti-human triple neurofilament MAb (1:100 dilution; Dakocytomation, Carpinteria, CA) were used for nerve fiber identification. The rest of the immunohisto-

TABLE 1. Clinical signs and results of IHC and WB in the tongues of positive animals

Outbreak and code	Clinical signs	IHC tongue[a]	WB tongue[a]	
			Epithelium	Muscle
A				
94151/1/7	No	+	+	+
81535/20	Yes	+	+	+
81535/82	No	+	+	−
101961/1	Yes	−	−	−
143486/1/03	Yes	−	−	−
143486/2/03	Yes	−	−	−
2595/1	No	+	+	+
10241/1/04	No	+	+	+
B				
120686/1/1	Yes	+	+	+
120686/1/2	Yes	+	+	+

[a] +, PrP^{SC} immunoreactivity; −, no Prp^{sc} immunoreactivity.

* Corresponding author. Mailing address: CEA-IZS Piemonte, Liguria e Valle d'Aosta, Via Bologna, 148 Turin 10154, Italy. Phone: 39 (0) 11 2686296. Fax: 39 (0) 11 2686322. E-mail: cea@izsto.it.

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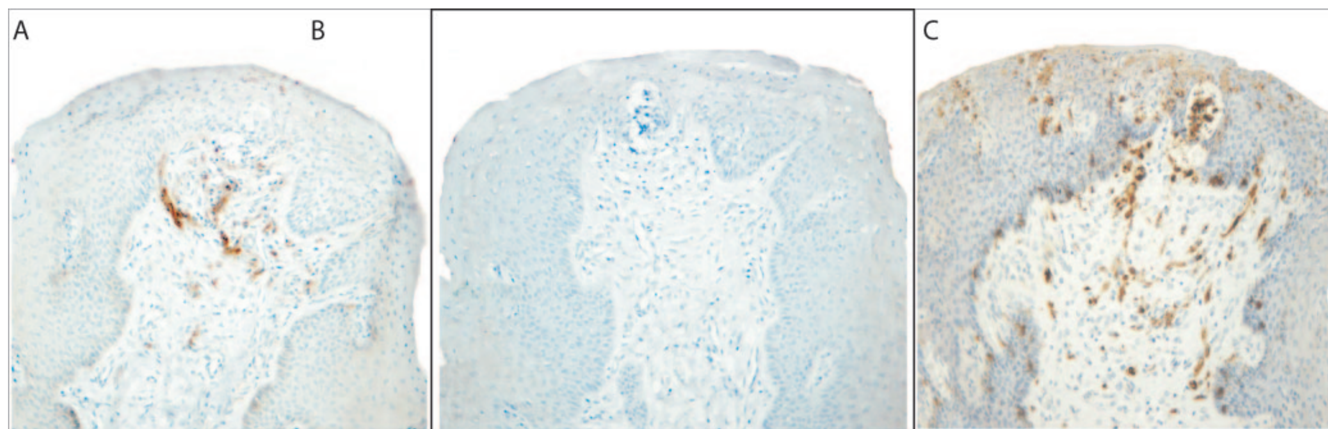

FIG. 1. PrP^{sc} immunostaining in the fungiform papilla of a sheep infected with naturally occurring scrapie (A), in the same papilla stained by PGP9.5 (C), and in the fungiform papilla of an unaffected sheep (negative control) (B). Magnification, ×20.

chemical procedure was carried out using a commercial immunoperoxidase technique (Vectastain ABC kit; Vector, Burlingame, CA), using 3,3′-diaminobenzidine (Dakocytomation, Carpinteria, CA) as chromogen; the sections were then counterstained with Meyer's hematoxylin.

The epithelium with underlying connective tissue and the muscle layer were separated from the frozen material and examined separately by a highly sensitive WB using sodium phosphotungstic acid precipitation (11). Briefly, 10% (wt/vol) homogenates from 0.2 to 0.4 g of frozen brainstem and tongue were precipitated with sodium phosphotungstic acid (Sigma) and then digested with proteinase K (50 μg/ml) at 37°C for 30 min. After denaturation, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) and electroblotted onto polyvinylidene difluoride membrane. The blots were incubated with MAb P4 (7) (1:5,000 dilution; R-Biopharm, Darmstadt, Germany). Immunoreaction was revealed with chemiluminescent substrate (Roche; 25 mM).

In flock A, PrP^{sc} was detected by WB and IHC in both areas of the tongues of five out of eight animals. Only one sheep showed clinical signs. No PrP^{sc} was detected in the tongues of the remaining three sheep, although they were at the clinical stage of the disease. In flock B, both animals contained PrP^{sc} in the tongue. (Table 1).

No immunoreaction was observed in the tongues of the 10 negative controls by either IHC or WB. PrP immunoreactivity was most prominent in the connective tissue core of the circumvallate and fungiform papillae, and it was occasionally observed in the basal region of the taste buds. PrP immunostaining showed a linear pattern corresponding to localization at the level of nerve processes, as revealed by anti-PGP 9.5 and anti-neurofilament immunostaining (Fig. 1).

PrP^{sc} was detected by WB in both the epithelium and the muscle layers of six out of seven cases. In one animal, the epithelium samples were positive but the muscle samples tested negative (Fig. 2). To estimate the relative concentration of PrP^{sc} in the tongues, we compared the intensities of the proteinase K-digested Western blot signals with calibration curves obtained by diluting the brainstems of the scrapie-positive sheep with a scrapie-negative tongue homogenate. The signal intensity of the positive tongues was generally similar to that obtained when 0.25 to 0.025 μg of a positive brainstem homogenate was diluted with 50 mg of a negative tongue homogenate. From this, we estimated that the levels of PrP^{sc} in the examined tongues were lower than those found in the

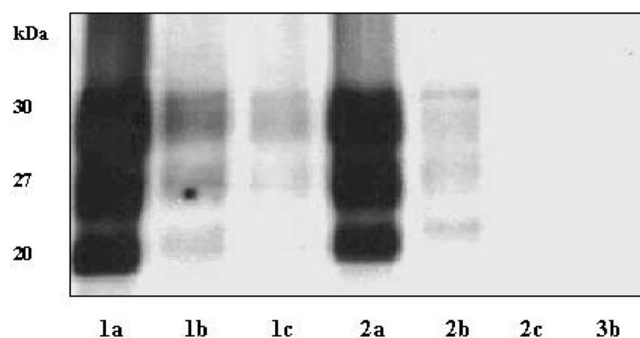

FIG. 2. Detection of PrP^{sc} in the brainstem and tongue by highly sensitive WB using sodium phosphotungstic acid precipitation. Lanes: 1, scrapie-positive sample (1a, brainstem; 1b, tongue epithelium; 1c, tongue muscle); 2, scrapie-positive sample (2a, brainstem; 2b, tongue epithelium; 2c, tongue muscle); 3b, tongue epithelium of a scrapie-negative sample.

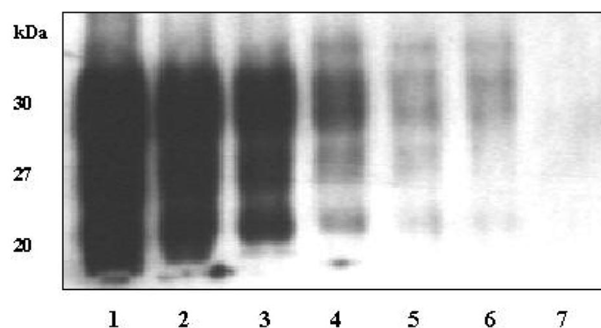

FIG. 3. WB of positive brainstem samples serially diluted in negative tongue homogenate: undiluted sample (lane 1), 10^{-1} (lane 2), 10^{-2} (lane 3), 10^{-3} (lane 4), 10^{-4} (lane 5), 10^{-5} (lane 6), and 10^{-6} (lane 7).

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corresponding brainstem by a factor of approximately 2×10^{-5} to 2×10^{-6} (Fig. 3).

To our knowledge, this is the first report of PrP^{sc} deposition detected in the lingual papillae of the tongues of sheep infected with naturally occurring scrapie. Further investigations are required to establish the onset of tongue involvement in the course of the disease. Although the rate of tongue involvement cannot be assessed yet, our findings suggest a need for revising the distribution of scrapie infectivity in sheep in peripheral tissues.

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