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PRNP sequences of Tibetan antelope, blue sheep and plateau pika from Qinghai-Tibet Plateau and the reactivities of their PrP proteins to rodent-adapted scrapie strains in RT-QuIC and PMCA

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Abstract

Background: Tibetan antelope (*Rhinopithecus*), blue sheep (*Pseudois nayauris*) and plateau pika (*Ochotona curzoniae*) are wild animals living in Qinghai-Tibet Plateau. Up to now, there is no report of naturally occurred TSE upon these animals. Their *PRNP* genes are not described in literature.

Methods: We obtained and sequenced the *PRNP* genes from 21 Tibetan antelopes, 4 blue sheep and 3 plateau pikas. Then we prepared their recombinant proteins. Using scrapie strains 263K, 139A, ME7 and S15 as the seeds, we tested the reactivities of the PrP proteins from sheep (rSheepPrP25-234) and pika (rPikaPrP23-230) in RT-QuIC. We also did the PMCA tests of the brain homogenates of domestic sheep and rabbit with the seeds of strains 263K and ME7.

Results: The *PRNP* genes of bovids were 771 bp long and encoded 256 amino acids (aa.), showing 100% homology with wild-type sheep PrP aa. sequence. The *PRNP* gene of pika was 759 bp long and encoded 252 amino acids, showing 92.1% homology with aa. sequence of domestic rabbit. Both the proteins of sheep and pika revealed positive reactions in 10^{-5} diluted seeds. Only rPikaPrP23-230 produced positive curves in 10^{-7} diluted seeds. PMCA tests ailed to produce PK-resistant PrP (PrP^{res}).

Conclusion: It is the first description of *PRNP* genes and PrP aa. sequences of those three animals in Qinghai-Tibet Plateau. In the presences of rodent prions, the PrPs of sheep and pika efficiently form fibrillation in RT-QuIC, but do not generate PrP^{res} in

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PMCA. Our results indicate that pika, as one of the important links in the biological chain in Qinghai-Tibet Plateau, may play an important role in prion circulation. And pika PrP deserves further analysis for its potential application value in the assays for human prion disease.

Keywords Tibetan antelope, Blue sheep, Lateau pika, *PRNP*, RT-QuIC, PMCA

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Introduction

Prion disease is a kind of transmissible spongiform encephalopathy (TSE) affected a species of mammals, i.e., Creutzfeldt-Jacob disease (CJD) in human, scrapie in sheet and goat, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer, etc [1, 2]. The etiologic agent for prion disease is prion, an abnormal conformational scrapie-like prion protein (PrP^{Sc}) converted from a normal host cellular form of prion protein (PrP^{C}) in central nervous system (CNS) [3]. The susceptibility, incubation period and cross-species transmission ability of scrapie are mainly determined by the host prion protein gene (*PRNP*) [4, 5].

Qinghai-Tibet Plateau is the highest plateau in the world with the average altitude of 4,000 meters. It is also the largest plateau in China with approximate 250 million km^2. As the animals under the national first-class state protection, Tibetan antelope (*Rhinopithecus*) is the only existing species of *Tibetan antelope* in the subfamily *Bovidae*, inhabiting the alpine grasslands and desert areas at an altitude of 3,250-5,500 meters, and mainly distributed in the Qinghai-Tibet Plateau centered in Qiangtang of China. Blue sheep (*Pseudois nayauris*) the only species of *blue sheep* in *Bovidae* subfamily, which can be divided into Sichuan subspecies and Xizang subspecies. It is the national second-class protected animals, mainly distributed in the Qinghai-Tibet Plateau and its adjacent mountain areas at an altitude of 3,200-5,000 meters, and their habitat environment is mostly in the areas of alpine bare rock and cliff. Plateau pika (*Ochotona curzoniae*) is a small non-hibernating herbivorous

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mammal, also known as black-lipped pika, belonging to the family *Pika* in the order *Rabiformes*. They mainly live in the areas of alpine meadow and alpine grassland with an altitude of 3,100-5,100 meters. Up to now, there is no report of naturally occurred TSE upon these animals. Their *PRNP* genes are not described in literature.

In the present study, we have, for the first time, obtained and sequenced the *PRNP* genes from the liver tissues of 21 Tibetan antelopes, 4 blue sheep and 3 plateau pikas who were natural death in the natural reserve region of Qinghai-Tibet Plateau. The *PRNP* genes of Tibetan antelope and blue sheep were 771 bp long and encoded 256 amino acids (aa.), showing 100% homology with wild-type sheep PrP aa. sequence. The *PRNP* gene of pika was 759 bp long and encoded 252 amino acids, showing relatively high homology (92.1%) in aa. sequence with that of rabbit. Furthermore, the full-length prokaryotic recombinant PrP proteins of sheep (rSheepPrP25-234) and pika (rPikaPrP23-230) were expressed and purified. Using the different dilutions of the brain homogenates of scrapie 263K-infected hamsters, scrapie 139A-, ME7- and S15-infected mice as the seeds, rSheepPrP25-234 and rPikaPrP23-230 were subjected into RT-QuIC assays, controlled by the full-length hamster PrP (rHaPrP23-231). Positive reactions were noted both in the preparations of rSheepPrP25-234 and rPikaPrP23-230, showing different reactogenicity. PMCA tests of the brain homogenates of domestic sheep and rabbit with the seeds of strains 263K and ME7 failed to produce PK-resistant PrP (PrP^{res}).

DOI: 10.55415/deep-2023-0005.v1

Materials and Methods

Ethics approval

The present study was approved by the Ethical Committee of the National Institute for Viral Disease Control and Prevention (Beijing, China) under the protocol 2009ZX10004-101, including the usages of the storage brain samples of scrapie agents infected rodents and liver tissues samples of Tibetan antelopes, blue sheep and plateau pikas.

PCR amplification and sequencing for PRNP

The liver tissues of 3 natural death *Ochotona curzoniae* (pika), 21 Tibetan antelopes, and 4 blue sheep were collected from Qinghai-Tibet Plateau in Qinghai province, China. Total DNAs were individually extracted with QIAamp DNA Mini Kit. The full-length *PRNP* sequence each sample was amplified by PCR technique using the different designed primers, including the primers for pika (upstream: 5'-ATGGCACACCTCAGCTACTGG-3'; downstream: 5'-TCATCCCACTATCAGGAAAA-3'), the primers for antelope and blue sheep (upstream: 5'-GCCACTGCTATACAGTCATTCA-3'; downstream: 5'-ACTACAGGGCTGCAGGTAGA-3'). The reaction conditions were 94°C for 1 min, 52°C for 30 s, 72°C for 40 s, totally 35 cycles. The PCR products were subjected to sequencing after purification according to the protocol described previously [6].

DOI: 10.55415/deep-2023-0005.v1

DNA and amino acid sequence alignment

The data of *PRNP* sequence and corresponding PrP amino acid sequence each tested sample were collated and analyzed with software DNAMAN 9.0. Comparisons of *PRNP* nucleotide sequences and amino acid sequences of pika, Tibetan antelope and blue sheep with those of other mammals were conducted with Clustal W method and DNAStar 7.0 software. The homology matrix and phylogenetic tree based on the amino acid sequence alignment were further constructed.

Construction of recombinant plasmids

Recombinant plasmid expressing full-length hamster PrP (rHaPrP23-231) was described previously [7]. To generate the recombinant plasmids expressing the full-length pika PrP (rPikaPrP23-230) and sheep PrP (rSheepPrP25-234), the *PRNP* sequence of sheep was obtained by PCR with the forward primer (5'-TTCCATATGAAGAAGCGACCAAAACCTGGC-3', with Nde-1 site) and the reverse primer (5'-CGGAATTCACTTGCCCCCCTTTGGTAAT-3', with EcoRI site), while the *PRNP* sequence of pika was obtained with the forward primer (5'-TTCCATATGAAGAAGCGGCCAAAACCCGGAG-3', with Nde-1 site) and the reverse primer (5'-CGGAATTCACTGGCCGCCCTCTGGT-3', with EcoRI site). The PCR products were cloned into T-vector. After verified by sequencing, the *PRNP* fragments were released from the cloning vectors with Nde-1/EcoRl digestion and inserted into a prokaryotic expressing vector pRSETA (cat. no. V35120; Thermo Fisher Scientific, Inc., Waltham, MA, USA), generating plasmids p-SheepPrP25-234

DOI: 10.55415/deep-2023-0005.v1

and p- PikaPrP23-230.

Protein expression and purification

Recombinant plasmids expressing rPikaPrP23-230, rSheep25-234 and rHaPrP23-231 were separately transformed into BL21(DE3) pLysS competent cells (cat. no. C1500; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). After treatment of the bacteria pellets with ultrasonication, the lysates were denatured with guanidine-HCl and the target proteins were purified by chromatography of Ni-NTA Superflow resin (cat. no. 30430; Qiagen, Hilden, Germany). The purified proteins were dialyzed into 10 mM sodium phosphate buffer (pH 5.8) and the concentrations of PrP proteins were adjusted to 500 μg/ml as determined by absorbance measured at 280 nm. Following filtration (0.22 μm syringe filter, cat. no. SLGP033RB, Merck millipore), the recombinant PrP proteins were aliquoted and stored at -80°C, respectively.

Brain samples of scrapie infected experimental rodents

The brain samples of hamsters inoculated intracerebrally with scrapie agent 263K, those of C57BL/6 (C57) mice inoculated intracerebrally with scrapie strains 139A, ME7 and S15, were enrolled in this study. The bioassay procedures, the clinical, neuropathological, and pathogenic features of these infected animals were described previously [8, 9]. The average incubation periods of 263K-infected hamsters, 139A-, ME7- and S15-infected mice were 80.1 ± 5.7, 183.9 ± 23.1 184.2 ±

DOI: 10.55415/deep-2023-0005.v1

11.8 and 175.4±1.0 days, respectively. Age-matched healthy hamsters and mice were used as control.

Preparation of brain homogenates

Brain homogenates were prepared according to the procedure described previously [9]. Brain tissues from the scrapie-infected and healthy rodents were washed in PBS for three times, and 10% (w/v) brain homogenates were prepared in cold lysis buffer (100 mM NaCl, 10mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.5) containing a mixture of protease inhibitors (Merck, 539134, US). The tissue debris were removed with low-speed centrifugation at 2000 *g* for 10 min and the supernatants were collected for further study.

Real-time quaking-induced conversion (RT-QuIC) assays

The details of the RT-QuIC assay were described previously[10]. Briefly RT-QuIC reaction contained 1 µl 10^{-5}, 10^{-7} and 10^{-9} diluted brain homogenates from various scrapie agents infected rodents, 1X PBS, 170 mM NaCl, 1 mM EDTA, 0.01 mM ThT, 0.001% SDS and 10 µg of rSheepPrP25-234, rPika23-230 or rHaPrP23-231 (concentration: 1.03, 1.17 and 0.6 mg/ml, respectively) in a final volume of 100 µl. Each sample was assayed in quadruplicated. The assay was conducted in a black 96-well, optical-bottomed plate (Nunc, 265301) on a BMG FLUOstar plate reader (BMG LABTECH). The main working conditions were fixed as follow: temperature, 50˚C; vibration speed, 900 rpm; vibration/incubation time, 90/30 sec; total reaction

DOI: 10.55415/deep-2023-0005.v1

time, 90 h. ThT fluorescence (excitation wavelength, 450 nm; emission wavelength, 480nm) each reaction was automatically measured every 30 min and expressed as relative fluorescence units (rfu). The cutoff value was set as the mean value of the negative controls plus 3 times the standard deviation. Each assay was repeated at least three times and Spearman-Karber analysis was used to estimate a seeding dose (SD_{50}). The SD_{50} was calculated using the following formula

$$xp = 1 + \frac{1}{2}d - dgp$$

xp=1 represents the highest log dilution giving all positive responses, d is the log dilution factor, p is the proportion positive at a given dose, and gp is the sum of values of p for xp=1 and all higher dilutions.

Protein misfolding cyclic amplification (PMCA)

PMCA was conducted in a sonicator (Misonix sonicator 3000 and 4000; Misonix, Farmingdale, NY, USA) containing an adjustable temperature water bath circulation system and a microplate horn for PCR tubes. The hamster-derived prion seed (from 263K-infected hamster brains) and mouse-derived prion seed (from ME7-infected mice brains) were diluted with the individual substrate of 10 % brain homogenates prepared from healthy hamsters or mice, respectively. 10 µl of different concentrations of seeds were mixed with 90 µl of substrate in thin-walled 0.2-ml PCR tubes containing 100 µl and placed in a floating position in the sonicator. One PMCA cycle included sonication process at PM 9.5 for 20 s and incubation process at 37 °C for 29 min and 40 s. In this study, a complete direct PMCA was 80 cycles for

DOI: 10.55415/deep-2023-0005.v1

mouse-derived prion seeds.

Western blots

Brain homogenates and PMCA products were separated by 12% SDS-PAGE and electronically transferred to nitrocellulose membranes with a Semi-dry facility. After blocking with 5 % nonfat-dried milk in TBS at 37°C for 1 h, the membranes were incubated at 4°C overnight with PrP-specific monoclonal antibody (mAb) SAF32 (1:5000 dilution, Cayman Chemical, Ann Arbor, MI, USA) at 37°C for 1h. After washing with TBST (containing 0.1 % Tween-20, pH7.6) for 4 times, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:5000 dilution). Immunoreactive signals were developed using an enhanced ECL kit (PE Applied Biosystems, Foster City, CA, USA).

DOI: 10.55415/deep-2023-0005.v1

Results

The PrP sequences of Tibetan antelope and blue sheep

Positive PCR products were obtained from 21 tested Tibetan antelopes and 4 blue sheep and sequencing assays revealed the *PRNP* genes were 771 bp long, which may encode 256 aa. long peptides (submitted to GenBank, MW194318 and MW194319, respectively). The *PRNP* sequences of 21 tested Tibetan antelopes were highly homologous, with only one nucleotide difference (C297T, 10:11) that was synonymous substitution. 4 tested blue sheep also showed high homology in *PRNP* sequences, with one synonymous substitution (A567G, 3:1).

The homology of *PRNP* sequences of Tibetan antelope, blue sheep and other mammals were analyzed and summarized in Fig. 1 a. Both Tibetan antelope (99.5 or 99.4%) and blue sheep (100% or 99.9%) showed highest homology in *PRNP* sequence with sheep. Higher *PRNP* sequence homologies of Tibetan antelope and blue sheep were also identified with goat, Thomson's gazelle, cattle and red deer, with homology over 90%. Less *PRNP* sequence homologies were observed with camel, dog, human, ferret and cat, while least with hamster, rabbit and mouse.

The homology of PrP aa. sequences among different mammals were showed in Fig. 1b. Tibetan antelope and blue sheep showed 100% identical in PrP aa. sequence with sheep, over 95% homology with goat (99.2%), Thomson's gazelle (99.2%), deer (98%) and cattle (97.7%), over 90% with camel (94.5%), dog (94.1%), ferret (93.8%), rabbit (92.9%), cat (91.4%), human (91.3%) and pika (90.5%), less than 90% with

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hamster (89.3%) and mouse (89.2%).

The PrP sequences of Ochotona curzoniae (pika)

Sequencing assays of the PCR products from the samples of 3 pikas showed the same *PRNP* sequence, with 759 bp long encoding 252 amino acids (submitted to GenBank, MT371369.1). As shown in Fig. 1a, pika showed the highest homology of *PRNP* gene with rabbit (90.4%). The homologies of *PRNP* genes of the other species animals and human were lower than 90%. Analysis of the homology of pika PrP aa. sequence with other species revealed the highest homology with rabbit (92.1%). Relatively higher homologies (over 90%) were observed in Thomson's gazelle (91.3%), Tibetan antelope (90.5%), blue sheep (90.5%), sheep (90.5%), camel (90.4%), red deer (90.1%) and cattle (90.1%) (Fig 1B).

Variations of PrP amino acids of Tibetan antelope, blue sheep and pika in comparison with other mammals

The exact PrP amino acid sequences from Tibetan antelope, blue sheep and pika, as well as other 13 species of mammals were shown in Fig. 2a. Variations of amino acids among various species mammals appeared in the regions of signal peptide, α-helix 3 and GPI anchor. There were 23 and 30 amino acids different between sheep and human, and between pika and human, respectively. Further, a phylogeny tree was constructed in order to illustrate the distances of PrP aa. sequences between various species (Fig. 2b). Tibetan antelope and blue sheep shared 100% homology in PrP aa.

DOI: 10.55415/deep-2023-0005.v1

sequence with sheep, and showed high homology with goat and other artiodactyls, i.e., Thomson's gazelle, deer and cattle, while apparently remote relationship with the animal species with naturally occurred prion disease (cat, camel and human) and routinely used experimental animals (hamster, mouse and ferret), as well as prion resistant animals (dog and rabbit). pika revealed relatively remote relationship of PrP aa. sequence with other species, including rabbit.

Hamster and mouse-adapted scrapie agents efficiently induce conversion of the recombinant blue sheep and pika PrPs in RT-QuIC

To test the feasibilities and reactivities of the PrPs from Tibetan antelope, blue sheep and pika in Qinghai-Tibet Plateau in RT-QuIC, full-length recombinant PrPs based on the *PRNP* sequences of blue sheep and pika were expressed and purified from *E. coli*. As the PrP aa. sequences of Tibetan antelope and blue sheep shared 100% homology, we only prepared the prokaryotic recombinant PrP blue sheep. SDS-PAGE and PrP-specific Western blot identified roughly a single band at the position of about 21 kDa in the preparations of rSheepPrP25-234 and rPikaPrP23-230 (Supp Fig. 1).

Same amounts of rSheepPrP25-234, rPikaPrP23-230 and rHaPrP23-231 were mixed with different prion strains at different dilutions (10^{-5}, 10^{-7} and 10^{-9}), and then subjected into RT-QuIC assays. Since rHaPrP23-231 was the routinely used substrate in our RT-QuIC assays, which revealed reliable reactivities in the reactions using the brain homogenates of hamsters and mice infected with various scrapie strains as the

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seeds at the dilutions over 10^{-7}, only the reactions of rHaPrP23-231 with 10^{-7} and 10^{-9} diluted scrapie strains were conducted. 10^{-5} diluted brain homogenate of normal hamster was used as negative control. In the preparation of 10^{-5} diluted scrapie strains, both rSheepPrP25-234 and rPikaPrP23-230 revealed positive reactive curves with all four tested scrapie strains, i.e., 263K (Fig. 3a), 139A (3b), ME7 (3c) and S15 (3d). In the preparation of 10^{-7} diluted scrapie strains, four wells of rSheepPrP25-234 showed positive curves with 263K, while only one well out of four displayed positive reaction after long lag with 139A and S15 and all four wells were negative with ME7. Contrarily, positive reactive curves were observed in all four wells of rPikaPrP23-230 with 263K, 139A and S15, and in 3 out of 4 wells with ME7. In parallel, positive curves were noticed in all reactions of rHaPrP23-231 with all four scrapie strains. In the preparation of 10^{-9} diluted scrapie strains, only one well of rHaPrP23-231 revealed positive curve with 139A, and the rest reactions were all negative. The SD_{50} values of agents 263K, 139A, ME7 and S15 in RT-QuIC using rSheepPrP25-234 as substrate were $10^{10.25\pm0.5}$/g, $10^{10.5\pm0.75}$/g, $10^{10\pm0.5}$/g and $10^{11.125\pm0.375}$/g, respectively, while those in RT-QuIC using rPikaPrP23-230 as substrate were $10^{16\pm0.75}$/g, $10^{12.25\pm0.5}$/g, $10^{12.63\pm0.13}$/g and $10^{12.38\pm0.38}$/g (Table 2).

The average lag times and the peaks of rfu of three species recombinant PrPs with four different scrapie strains at dilutions of 10^{-5} and 10^{-7} were summarized in Table 1. Both rSheepPrP25-234 and rpikaPrP23-230 were positive in 10^{-5} dilutions of all strains and reached to the rfu detecting limitation (260,000) of our RT-QuIC. However, the lag times of rSheepPrP25-234 were obviously longer than that of

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rpikaPrP23-230. Contrary to rSheepPrP25-234 that showed RT-QuIC positive only in the dilution of 10^{-7} of 263K with relatively long lag phase (24.3h), both rpikaPrP23-230 and rHaPrP23-231 were elicited positive reactions in the presences of 10^{-7} diluted those four scrapie agents. Moreover, rpika23-230 showed notably shorter lag times and higher peaks of rfu than rHaPrP23-231 in the reactions with all tested four scrapie strains.

Hamster- and mouse-adapted scrapie agents fails to induce conversion of the native brain PrP^C from sheep and rabbit in PMCA

As Tibetan antelope, blue sheep and plateau pika are national protected wild animals, it is completely impossible to obtain their fresh brain tissues. To evaluate the possible conversion activities of mouse- and hamster-adapted scrapie agents on the brain PrP^C of those plateau animals in PMCA, the brain homogenates of domestic sheep and rabbit were prepared and used as the mimics. As expected, clear PK-resistant PrP signals (PrP^{res}) were observed in the reactions using agent 263K as seed and hamster brain homogenate as substrate (10^{-4}) (Fig. 4a, upper panel) and that using agent ME7 as seed and mouse brain homogenate as substrate (10^{-3}) (4b, upper panel), respectively. However, under our experimental condition, no PrP^{res} signal was detected in any reaction of agent 263K (Fig. 4a, lower panel) or ME7 (4b, lower panel) mixed with brain homogenates of sheep (left) or rabbit (right). Further two rounds of blind passages of the PMCA products to the brain homogenates of sheep and rabbit in PMCA still failed to identify PrP^{res} signal. It implies that the prions in the brain

DOI: 10.55415/deep-2023-0005.v1

tissues of scrapie infected experimental hamster and mouse are incapable of inducing conversion the brain PrP^C in domestic sheep and rabbit by PMCA.

Discussion

In the current study, we have, for the first time, reported the *PRNP* sequences of three wild mammals in Qinghai-Tibet Plateau, Tibetan antelope (*Rhinopithecus*), blue sheep (*Pseudois nayauris*) and plateau pika (*Ochotona curzoniae*). Both Tibetan antelope and blue sheep reveal extremely high homology in *PRNP* gene and 100% identical in PrP aa. sequences with sheep. Two novel synonymous polymorphisms have been identified, i.e., C297T in Tibetan Antelope and A567G in blue sheep, involving in the encoding codon for G66 in the 1st octarepeat and N189 in the 2nd α-helix. No polymorphism is found in 3 pikas' samples. Because of the limited numbers of blue sheep and pikas in this study, presence of other polymorphism in the *PRNP* genes of those species cannot be absolutely excluded.

It is well-known that the polymorphisms at codons 136, 154 and 171 in sheep PrP are closely associated with the susceptibility to scrapie, among them the type of VRQ is highly sensitive, type of ARQ is moderate sensitive and type of ARR is less sensitive [11-14]. All tested samples of Tibetan antelope and blue sheep in this study display ARQ genotype at codons 136, 154 and 171. It highlights that those two species animals are potentially susceptible to scrapie. We must admit that the conclusion of the genotype of ARQ in Tibetan antelope and blue sheep is based on the study with the limited number of the tested individuals that was obtained from the

DOI: 10.55415/deep-2023-0005.v1

limited regions. It cannot be extended to the whole population in Qinghai-Tibet Plateau.

Plateau pika is a species endemic to Qinghai-Tibet Plateau with a large number. Plateau pika shows very high homology with American pika (*Ochotona princeps*) in *PRNP* gene but relatively remote association with other tested mammals, including rabbit with the highest homology (92.1%). The susceptibility of pika to scrapie or other prion agents remains unknown, even hard to predict. Plateau pika is considered to play an important role in maintaining biodiversity and ecosystem balance in Qinghai-Tibet Plateau where there are about 210 wild mammal species. Numerous caves pikas excavated provide nests not only for themselves but also for many other small birds and lizards. Pikas' caves nourish the plants and provide ideal condition for the diversity of plant species in this region. Meanwhile, pika is also the main prey of most small and medium-sized carnivores and almost all raptors on the grassland. As one of the important links in the biological chain in Qinghai-Tibet Plateau, one may assume the importance in prion circulation if pika is susceptible to prion infection.

Our data here verify that the full-length recombinant PrP proteins of both sheep and pika can be efficiently converted and show positive in the RT-QuIC assays in the presences of various hamster- and mouse-adapted scrapie strains, which indicates the PrP proteins of blue sheep, Tibetan antelope and plateau pika are able to form fibrous structures by hamster's and mouse's prions under the RT-QuIC experimental condition. It is known that the efficient transmission between species depends largely, but not absolutely, on the homology of the host PrP proteins between donor and

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recipient [3, 15, 16]. Such phenomenon may also appear, to certain extent, on the *in vitro* tests for misfolding proteins, such as RT-QuIC and PMCA [17, 18]. However, breakthrough of species barrier is much easier under the conditions of RT-QuIC and PMCA [18]. The RT-QuIC results here verify again a certain of breakthrough of species barrier, despite of apparent difference in PrP aa. sequences of sheep and pika in comparison with those of the doners (hamster and mouse).

We also find the difference in the RT-QuIC reactivities to three mouse-adapted scrapie strains between sheep and pika PrP proteins. Pika recombinant PrP shows apparently higher RT-QuIC reactivity than sheep PrP. Even, pika PrP displays slightly stronger reactivity than hamster PrP under our experimental condition. It is understandable that the sheep recombinant PrP does not have stronger RT-QuIC reactivity to the scrapie strains used in this study, because all prion strains here are rodent-adapted scrapie ones. Pika and sheep PrPs show the similar percentages of homology compared with mouse and hamster. Besides of the regions of signal peptide and GPI anchor that are removed in the full-length recombinant proteins, the most obvious diversity of aa. sequence between those two species locates in the region of 3rd α-helix. The exact molecular mechanism needs further exploration. Nevertheless, the good and stable reactivity of pika PrP to various rodent-adapted scrapie agents in RT-QuIC deserves further analysis for its feasibility as the substrate in RT-QuIC tests, even in the assays for human prion disease.

Generally, PMCA products possess both biochemical characteristics and infectivity of prions, whereas RT-QuIC products are more of a reflection of the

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fibrillation of prions [19-21], which usually lack infectivity [22]. Overcome of natural species barrier of prion infection is observable in the experimental bioassays of inoculation of PMCA products. Our previous study has confirmed that inoculation of the newly formed cross-species PMCA products of hamster (strain 263K)- and mouse-adapted (strain 139A) scrapie agents amplified with heterogeneous brain tissues, 139A-hamster PMCA-PrP^{res} and 263K-mouse PMCA-PrP^{res}, induce experimental transmissible spongiform encephalopathies (TSE) on opposite animals. On the contrary, the homologous PMCA products (139A-mouse PMCA-PrP^{res} and 263K-hamster PMCA-PrP^{res}) showed no infectivity against the opposite animals [23]. Easily overcoming species barrier of prion infection by PMCA are repeatedly documented in order to understand the transmission between different species animals and from animals to human [24]. With the help of PMCA, PrP^{res} can be generated in the reactions of different species of PrP^{Sc} with PrP^{C} from rabbit and dog that are respectively considered as very poorly susceptible and tolerant to prion infection [25, 26]. It strongly highlights that PMCA can help prion strains to breakthrough a certain of species barrier and propagate both *in vitro* and *in vivo*. However, three blind passages of hamster and mouse prions in this study failed to induce the conversion onto the brain homogenates of domestic sheep and rabbit in PMCA. One direct technical possibility is the relatively limited amplifying rounds and times in PMCA. Further increase of PMCA rounds is deserved. On the other hand, high efficiency of PMCA in circumventing species barriers make people doubt that the species barriers in real world is potentially underestimated, and therefore, careful interpretation of the

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results of PMCA experiments is required [24]. Nevertheless, numerous species of ruminants are verified to be susceptible to the infection of scrapie and BSE [3, 27-29]. One may assume that Tibetan antelope and blue sheep, showing high homology in their PrP aa. sequences with sheep, are also susceptible to the infection of natural scrapie.

In conclusion, this study is the first description of *PRNP* genes and PrP aa. sequences of those three animals in Qinghai-Tibet Plateau. In the presences of rodent prions, the PrPs of sheep and pika efficiently form fibrillation in RT-QuIC, but do not generate PrP^{res} in PMCA. Our results indicate that pika, as one of the important links in the biological chain in Qinghai-Tibet Plateau, may play an important role in prion circulation. And pika PrP deserves further analysis for its potential application value in the assays for human prion disease.

Conflict of Interest

The authors declare that they have no conflicts of interest and consent for publication.

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Figure legends

Figure. 1 Homology matrixes of the sequences of *PRNP* gene and amino acid of PrP proteins of various species of mammals and human. **a** DNA sequences. **b** Amino acid sequences.

Figure. 2 Comparison of the variations of amino acids of PrP proteins of various mammals and human. **a** Full-length amino acid sequences of PrPs. The various functional and secondary structural regions within PrP sequences are indicated with colours. The amino acid numbers are shown on the right. **b** Phylogenetic tree based on amino acid sequences of PrPs.

Figure. 3 RT-QuIC assays of rSheepPrP25-234, rpikaPrP23-230 and rHaPrP23-231 to four different rodent-adapted scrapie strains. **a** hamster-adapted strain 263K. **b** mouse-adapted strain 139A. **c** mouse-adapted strain ME7. **d** mouse-adapted strain S15. The dilutions of scrapie strains are shown in the top each graph. Neg Ctrl: negative control of 10^{-5} diluted brain homogenate of normal hamster. Blank Ctrl: blank control of PBS. Various recombinant PrP proteins are indicated on the right.

Figure. 4 PMCA tests of the brain homogenates of domestic sheep and rabbit with two rodent-adapted scrapie strains. **a** hamster-adapted scrapie strain 263K. **b** mouse-adapted strain 139A. Above panels are the PMCA tests with homologous brain substrate and seed. The PMCA products were subjected into PrP-specific Western

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blots with (+) or without (-) digestion of proteinase K (PK). BH: brain homogenate.

Supplemental Figure. 1 Analyses for the purified recombinant PrP proteins based on the PRNP sequences of blue sheep and plateau pika. **a** SDS-PAGE. **b** Western blot. The molecular weights are indicated on the left.

DOI: 10.55415/deep-2023-0005.v1

Table. 1 The average lag times and the peaks of rfu of three species recombinant PrPs with 4 scrapie strains at dilutions of 10^{-5} and 10^{-7}

PrP protein	Dilution	10^{-5}				10^{-7}			
	Strain	263K	139A	ME7	S15	263K	139A	ME7	S15
rSheepPrP25-234	Average lag time (h)	13.8±0.9	14.2±4.3	21.9±16.8	10.9±1.9	23.6±12.8	-	-	-
	Peak of rfu (X1000)	260	260	260	260	260	-	-	-
rPikaPrP23-230	Average lag time (h)	4.4±0.2	5.2±2.5	4.3±0.3	3.8±0.6	12.9±3.8	6.0±1.4	13. 3±6	18.6±12.4
	Peak of rfu (X1000)	260	260	260	260	260	260	260	260
rHaPrP23-231	Average lag time (h)	ND	ND	ND	ND	19.3±8.7	8.6±1.7	17.5±8.8	23.7±18.5
	Peak of rfu (X1000)	ND	ND	ND	ND	204±9.1	165±10.0	211±17.4	181±8.0

ND: not done

Table. 2 The average SD_{50} values of three species recombinant PrPs with 4 scrapie strains

DOI: 10.55415/deep-2023-0005.v1

PrP protein	SD_{50}			
Strain	263K	139A	ME7	S15
rSheepPrP25-234	$10^{10.25\pm0.5}$/g	$10^{10.5\pm0.75}$/g	$10^{10\pm0.5}$/g	$10^{11.125\pm0.375}$/g
rPikaPrP23-230	$10^{16\pm0.75}$/g	$10^{12.25\pm0.5}$/g	$10^{11.5\pm0.5}$/g	$10^{12.375\pm0.375}$/g
rHaPrP23-231	$10^{12.63\pm0.13}$/g	$10^{12.25\pm0.25}$/g	$10^{12.625\pm0.125}$/g	$10^{12.625\pm0.126}$/g

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