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A B S T R A C T

In this study, we examined in Sardinia the brain of 555 autochthonous sheep, 50 goats, and 4 mouflons which were found affected by neurological signs. We found 6 goats and one mouflon with meningoencephalitis caused by Cryptococcus sp. There was no evidence of cryptococcal infections in any ofthe examined sheep. MLST genotyping on Cryptococcus sp. isolates identified Cryptococcus gatti genotype AFLP4/VGI and Cryptococcus neoformans var. neoformans genotype AFLP2/VNIV. Phylogenetically, all Cryptococcus gattii isolates fell within the autochthonous animal, human and environmental Mediterranean isolate cluster, forming a distinct branch along with environmental strains from Alicante, in the southern Mediterranean coast of Spain.

1. INTRODUCTION

Although the majority of Cryptococcus species are considered to be non-pathogenic, Cryptococcus neoformans and Cryptococcus gattii are worldwide pathogens which cause granulomatous inflammation in many organs in humans and animals (Springer and Chaturvedi, 2010; McGill et al., 2009). C. neoformans (serotypes A and D) is a major opportunistic pathogen among the increasing

number of immune-compromised individuals, whereas C. gattii(serotypesBandC)is gainingprominence as aprimary cause of diseases (Hagen et al., 2012). After being believed tobe restrictedto tropical and sub-tropical areas,inthepast decade C. gattii has been diagnosed in many temperate areas across the world, including Europe (Springer and Chaturvedi, 2010; Kidd et al., 2004). This new epidemiological situation has increased awareness of cryptococcal infections and underscored the need for more precise classification of the isolates for a better understanding of how it is spreading worldwide and evolving to be a lethal pathogen. As far as genotyping is concerned, multilocus sequence typing (MLST) is widely recognized as the method of choice for strain typing (Meyer et al., 2009). In light of the current epidemiological situation, a precise molecular characterization of the Cryptococcus isolate circulating in livestock is of interest because animals play an important role in signaling and spreading Cryptococcus sp. in a given region (Springer and Chaturvedi, 2010). Here we investigated the occurrence of this yeast Cryptococcus sp. in neurologically sick autochthonous small ruminants of Sardinia (Italy).

2. MATERIALS AND METHODS

Taking advantage of passive diagnostic surveillance activity for nervous diseases, which was established to control Transmissible Spongiform Encephalopathies (TSEs) in autochthonous small ruminants in Sardinia, over the 1994–2011 period we had the opportunity to examine the brains of 555 sheep, 50 goats and 4 mouflons showing signs of neurological disorders by histological, bacteriological and virological diagnostic means. The brains were collected at the necroscopy and then divided into the two hemispheres by a medial cutting. From one of brain hemisphere, attempts to isolate bacteria and fungi were made by inoculating fresh tissue taken from different areas into solid Sheep Blood Agar medium, and incubating the plates aerobically at 37 8C for 48 h. The same nervous tissues samples were streaked for isolation of fungi on Sabouraud dextrose agar plates, which were incubated at 37 8C for up to 10 days. Mucoid and creamy colonies indicative of Cryptococcus

sp. were examined by Gram staining for typical microscopic morphology and then confirmed by biochemical tests (API 20C Aux system), according to the manufacturer's instructions. Biochemical profiles were interpreted by using the Api web database software (BioMe´ rieux, Marcy l'Etoile, France). After being identificated, the colonies of Cryptococcus sp. were stored at 80 8C in conventional medium. The other brain hemisphere was fixed in 10% neutral buffered formalin for 15 days and transverse sections were then taken at different levels, including the obex, caudally to the cerebellar peduncles, superior colliculus, between the medial and lateral geniculatus bodies, hypothalamus and basal nuclei. These sections, after being embedded in paraffin by conventional procedures, were cut into 5mmthick slices and stained with hematoxylin and eosin for histological examination. For genotyping, the DNA from all the Cryptococcus sp. found in the affected brains were submitted to MLST analysis, which was conducted by amplifying and sequencing 10 chromosomal loci, namely CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, URA5, CAP10, MPD1, and TEF1a according to previously described methods (Hagen et al., 2012; Meyer et al., 2009). Consensus sequences were assembled and alignments were generated by using MEGA version 5.1 and BioEdit version 7.2.5 (Tamura et al., 2011). The concatenated alignments were analyzed using the Neighbor-Joining method with modelKimura 2 parameters (MEGA 5.1). Bootstrap analysis with 1000 replicates was used to determine the significance of branches. Furthermore established CD immunophenotyping of inflammatory cells (Maestrale et al., 2013) was performed in paraffin embedded brain sections to define the local immune response related to Cryptococcus sp. In addition according to previously published methods (Krockenberger et al., 2001), an in situ serotyping was performed using monoclonal antibodies (mAb) which bind to different glucuronoxylomannan (GXM) epitopes shared by the cryptococcal serotypes. MAb 471 is reactive with all four Cryptococcus serotypes (C. gattii and C. neoformans) whereas mAbs 302 and 1326 are species specific, only reacting with serotypes A and D (C. neoformans); reactivity with mAb 471 and an absence of reactivity with mAbs 302 and1326 is indicative of C. gattii.

3. RESULTS

Among the examined small ruminants, in addition to observing several other neuropathological disorders, we found 6 goats and one mouflon affected by Cryptococcus sp. This diagnosis was confirmed by histopathology and isolation on Sabouraud's dextrose agar culture. There was no evidence of cryptococcal infections in any of the examined sheep. When compared with reference strains, 5 Cryptococcus isolates belonging to 5 out of the 6 affected goats matched the C. gattii strain WM179, serotype B, AFLP4/VGI genotype (bootstrap support value of 100). The remaining caprine isolate and that from the mouflon were consistent with C. neoformans strain WM629, serotype D (variety neoformans) AFLP2/VNIV genotype (bootstrap support value of 100). During the phylogenetic analysis, our C. gattii isolates clustered with VGI/AFLP4 Mediterranean isolates after comparison with previously published human, animal, and environmental European isolates whose sequences have been deposited in the NCBI and CBS-KNAW Fungal Biodiversity Centre genome sequence databases (Fig. 1). However, they were demonstrated to fall within the same cluster as two Spanish environmental isolates from Alicante, in the southern Mediterranean coast of Spain (bootstrap support value of 77) (Colom et al., 2012) (Fig. 1). Mating-typing by amplification of either the STE12a or STE12a genes (Hagen et al., 2012) revealed that the 5 C. gattii and the 2 C. neoformans var. neoformans strains were MATa and MATa, respectively. Histologically, regardless of the Cryptococcus sp. involved, the brains exhibited lesion patterns indicative of non-suppurative meningoencephalitis with the presence of aggregates of Cryptococcus organisms causing diffuse perivascular ‘‘soap bubble’’ changes predominantly scattered throughout the basal nuclei, mesencephalon and meninges. By CD cell immunophenotyping methods, a moderate mononuclear cell infiltrate was observed within the lesions or organized as perivascular cuffing with presence of CD163+ macrophages, CD3+ T cells, and CD79+ B cells. A comparison of the intensity of the immunohistochemical staining suggested that the different CD epitopes were equally represented (Fig. 2). When brain tissue sections were probed for in situ serotyping, antibodies showed a binding pattern consistent with 5 C. gattii (mAb 471—positive, mAbs 302 and 1326—negative) and 2 C.

neoformans (mAb 471—positive, mAbs 302 and 1326—positive), confirmatory of the MLST genotyping results (Fig. 3 and Table 1).

4. DISCUSSION

Our results suggest that C. gattii has been the major source of neurological cryptococcosis in small ruminants in Sardinia over the last decade, with C. neoformans var. neoformans found only sporadically prior to 2004. Finding C. gatti in neurologically affected Sardinian goats supports the theory which argues that C. gatti is a new emerging pathogen, with ongoing outbreaks in Canada and US Pacific Northwest states since 1999 (Kidd et al., 2004). Interestingly, Italy's case of C. gattii infection in a goat was in 2003, suggesting that expansion of the range of C. gattii over the last decade has occurred contemporarily in the temperate climate regions, even at wide geographic distances. Genotyping of all isolates demonstrated that in Sardinia there is only one strain of C. gattii, which clustered more closely with environmental isolates from Alicante in the southern Mediterranean coast of Spain (Colom et al., 2012) than with those from other regions of Italy (Hagen et al., 2012). A typical goat breed, termed Murciana, is raised in the Murcia region and extensively exported across the Mediterranean area, including Sardinia. We speculate that these goats may have played a role as asymptomatic carriers, thus explaining the presence of this distinct molecular type of C. gatti in Sardinia. In our survey, no neurological disorders due to Cryptococcus gattii or other Cryptococcus sp. were found in sheep, though they were by far the most represented animal examined. This finding, coupled with the fact that the sheep and goats shared the same environment, might indicate that goats are more susceptible to cryptococcosis. However, we cannot rule out the possibility that their different environmental habits may make goats more aptto coming in close contact with sources of Cryptococcus. Similar results appear to have been reported in Spain, where the majority of the C. gattii strains analyzed in molecular epidemiology studies were from goats (Colom et al., 2012). Collectively, these results indicate that C. gattii detection in goats could act as a significant gauge to

help researchers identify high-risk areas for infections. Based on immunohistopathological evidence, both Cryptococcus species found here can be considered primary etiological agents of meningoencephalitis in small ruminants. In fact, immunohistochemical staining showed a massive presence of macrophages as well as T and B lymphocytes, indicating ahost reaction against Cryptococcus further research is needed to establish the real range of this yeast as an agent of animal disease in Italy (Danesi et al., 2014). Notably, here we confirm that in situ serotyping is a useful tool in retrospective studies aimed to distinguish C. gattii from C. neoformans.

ACKNOWLEDGEMENTS

The authors would like to thank Alfio Lai for technical assistance. This work was supported by the IZS SA 01/11 and Ricerca Finalizzata 2010 grants from Italian Ministero della Salute.

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Figures

Fig. 1. Neighbor-joining tree of Sardinian C. gattii isolates and published European isolates. The published sequences of the European isolates have been deposited in the NCBI and CBS-KNAW Fungal Biodiversity Centre genome sequence databases. The tree was constructed by using MLST genotyping data from 10 unlinked loci. The Sardinian C. gattii isolates fell within the autochthonous animal, human and environmental Mediterranean isolate cluster, forming a distinct environmental branch with environmental strains from Alicante, in the southern Mediterranean coast of Spain.

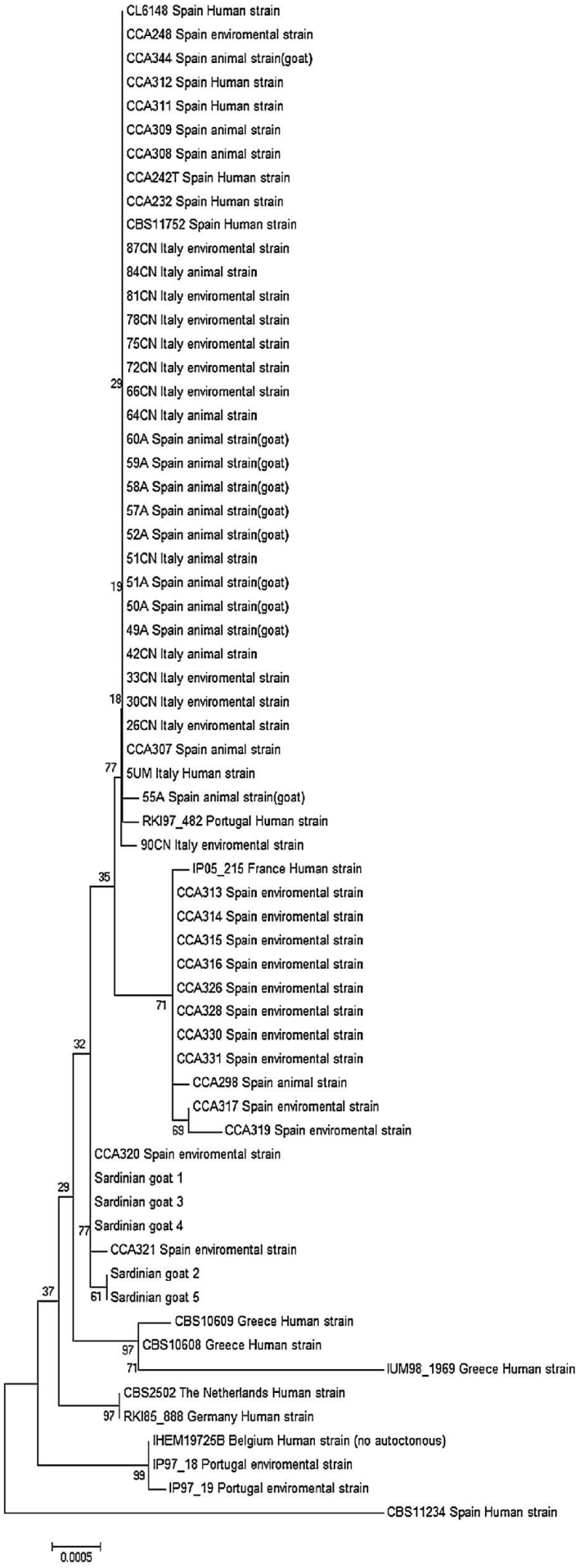
CL6148 Spain Human strain
CCA248 Spain enviromental strain
CCA344 Spain animal strain(goat)
CCA312 Spain Human strain
CCA311 Spain Human strain
CCA309 Spain animal strain
CCA308 Spain animal strain
CCA242T Spain Human strain
CCA232 Spain Human strain
CBS11752 Spain Human strain
87CN Italy enviromental strain
84CN Italy animal strain
81CN Italy enviromental strain
78CN Italy enviromental strain
75CN Italy enviromental strain
72CN Italy enviromental strain
29
66CN Italy enviromental strain
64CN Italy animal strain
60A Spain animal strain(goat)
59A Spain animal strain(goat)
58A Spain animal strain(goat)
57A Spain animal strain(goat)
52A Spain animal strain(goat)
51CN Italy animal strain
19
51A Spain animal strain(goat)
50A Spain animal strain(goat)
49A Spain animal strain(goat)
42CN Italy animal strain
33CN Italy enviromental strain
18
30CN Italy enviromental strain
26CN Italy enviromental strain
CCA307 Spain animal strain
77
5UM Italy Human strain
55A Spain animal strain(goat)
RKI97_482 Portugal Human strain
90CN Italy enviromental strain
35
IP05_215 France Human strain
CCA313 Spain enviromental strain
CCA314 Spain enviromental strain
CCA315 Spain enviromental strain
CCA316 Spain enviromental strain
CCA326 Spain enviromental strain
71
CCA328 Spain enviromental strain
CCA330 Spain enviromental strain
32
CCA331 Spain enviromental strain
CCA298 Spain animal strain
CCA317 Spain enviromental strain
69
CCA319 Spain enviromental strain
CCA320 Spain enviromental strain
Sardinian goat 1
29
Sardinian goat 3
Sardinian goat 4
77
CCA321 Spain enviromental strain
Sardinian goat 2
37
61
Sardinian goat 5
CBS10609 Greece Human strain
97
CBS10608 Greece Human strain
71
IUM98_1969 Greece Human strain
CBS2502 The Netherlands Human strain
97
RKI85_888 Germany Human strain
IHEM19725B Belgium Human strain (no autoctonous)
IP97_18 Portugal enviromental strain
99
IP97_19 Portugal enviromental strain
CBS11234 Spain Human strain
0.0005

Fig. 2. Pathological changes with lymphocyte and macrophage infiltration in Cryptococcus sp. affected goat brains. (A) Severe non-suppurative meningoencephalitis with the presence of aggregates of Cryptococcus organisms (haematoxylin-eosin). (B–D) CD immune-phenotyping reveals presence of CD3+ T cells, CD163+ macrophages and CD79+ B cells, respectively, in serial paraffin-embedded sections of goat brains affected by C. gattii. Immune reactions were visualized by 3-30 -diaminobenzidine (DAB) chromogen. Mayer's hematoxylin counterstain, scale bars 100mm

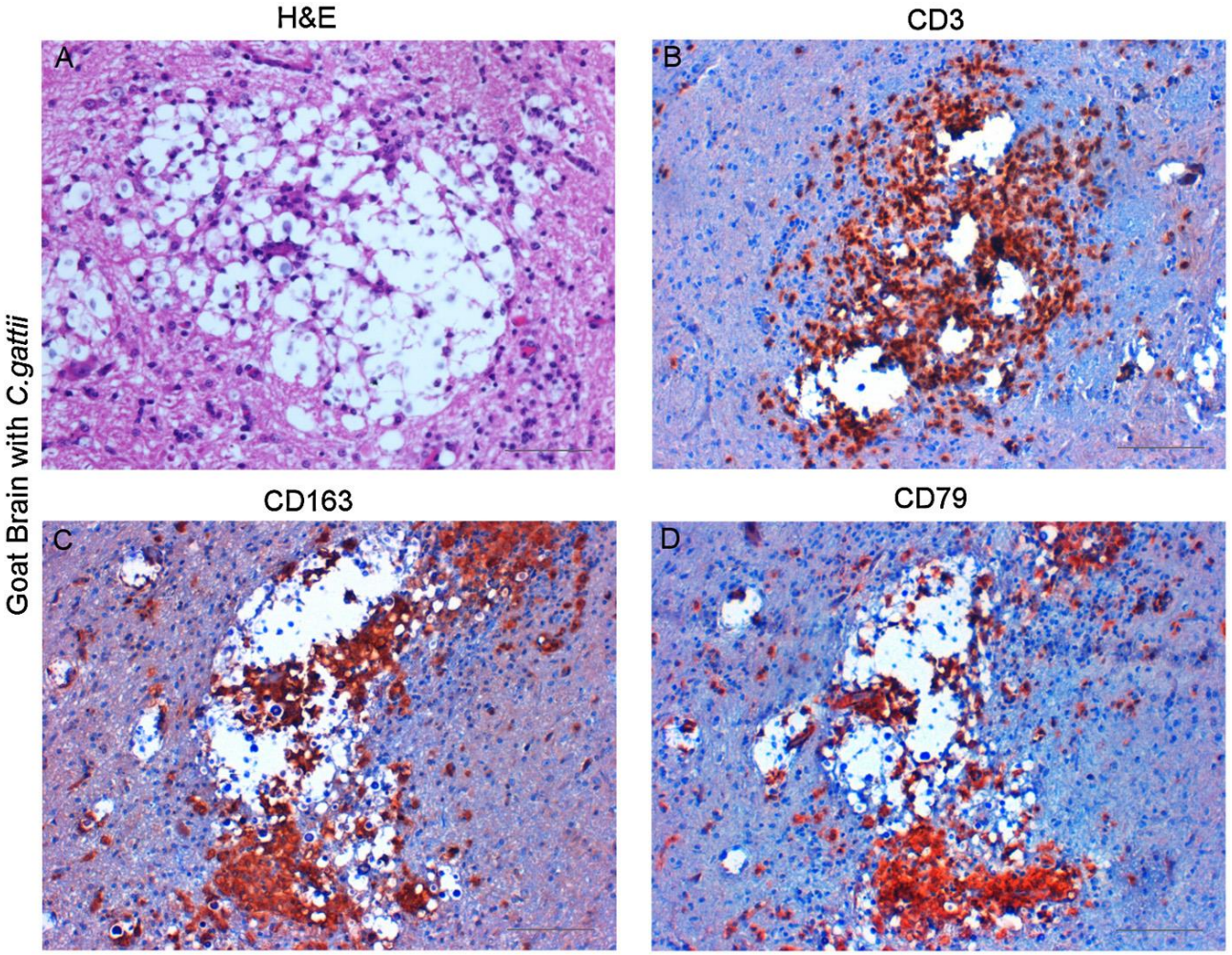

Fig. 3. In situ serotyping of Cryptococcus sp. affecting goat brains. (A) C. gattii labeled by Mab 471. (B and C) Both Mab 302 and Mab 1326 are not reactive with C. gattii, respectively. (D–F) Mab 471,

Mab 302 and Mab 1326 bind to C. neoformans. Immune reactions were visualized by 4-chloro-2-methylbenzenediazonium chromogen. Mayer's hematoxylin counterstain, scale bars 100mm.

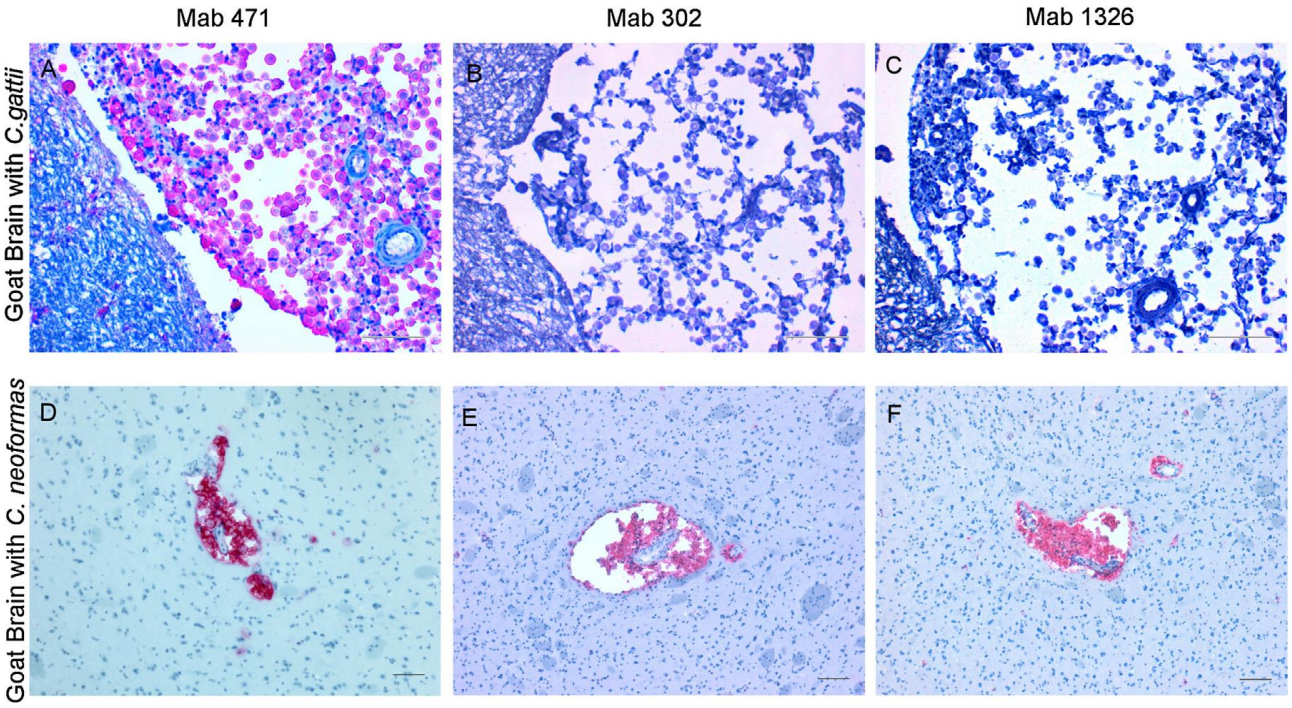

Table 1 In situ serotyping and MLST genotyping results from small ruminants with neurological cryptococcosis. Animal species Year In situ serotyping MLST genotyping Goat 1994 C. neoformans C. neoformans v. neoformans Goat 2003 C. gattii C. gattii Mouflon 2004 C. neoformans C. neoformans v. neoformans Goat 2007 C. gattii C. gattii Goat 2009 C. gattii C. gattii Goat 2010 C. gattii C. gattii Goat 2011 C. gattii C. gattii

Animal species	Year	*In situ* serotyping	MLST genotyping
Goat	1994	*C. neoformans*	*C. neoformans v. neoformans*
Goat	2003	*C. gattii*	*C. gattii*
Mouflon	2004	*C. neoformans*	*C. neoformans v. neoformans*
Goat	2007	*C. gattii*	*C. gattii*
Goat	2009	*C. gattii*	*C. gattii*
Goat	2010	*C. gattii*	*C. gattii*
Goat	2011	*C. gattii*	*C. gattii*