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Genetic and pathological characteristics of *Cryptococcus gattii* and *Cryptococcus neoformans* var. *neoformans* from meningoencephalitis in autochthonous goats and mouflons, Sardinia, Italy

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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 of the examined sheep. MLST genotyping on *Cryptococcus* sp. isolates identified *Cryptococcus gattii* 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* (serotypes B and C) is gaining prominence as a primary cause of diseases (Hagen et al., 2012). After being believed to be restricted to tropical and sub-tropical areas, in the past 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 necropsy 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 °C for 48 h. The same nervous tissues samples were streaked for isolation of fungi on Sabouraud dextrose agar plates, which were incubated at 37 °C for up to 10 days. Mucoïd 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 (BioMérieux, Marcy l'Etoile, France). After being identified, the colonies of *Cryptococcus* sp. were stored at 80 °C 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 geniculate bodies, hypothalamus and basal nuclei. These sections, after being embedded in paraffin by conventional procedures, were cut into 5mm thick 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 model Kimura 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 (Maestrone 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 and 1326 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. gattii* in neurologically affected Sardinian goats supports the theory which argues that *C. gattii* 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. gattii* 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 apt to 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 a host 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*.

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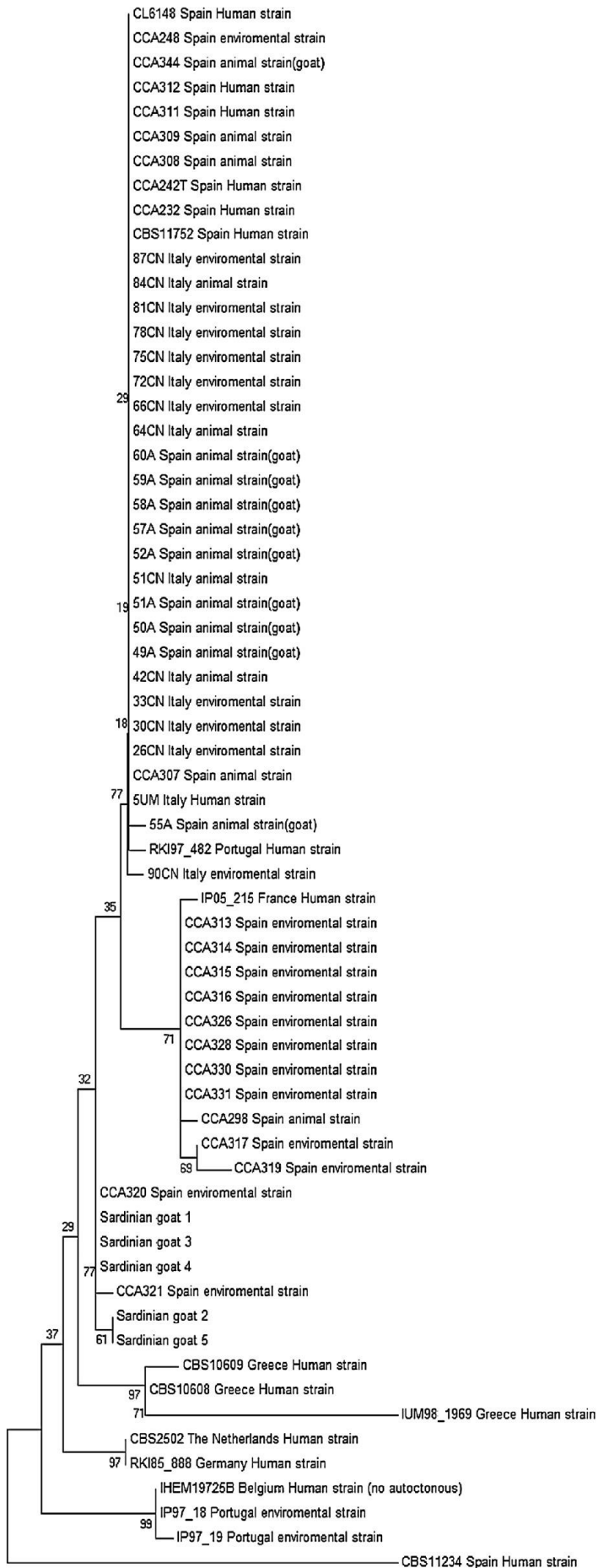
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Figures

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.



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

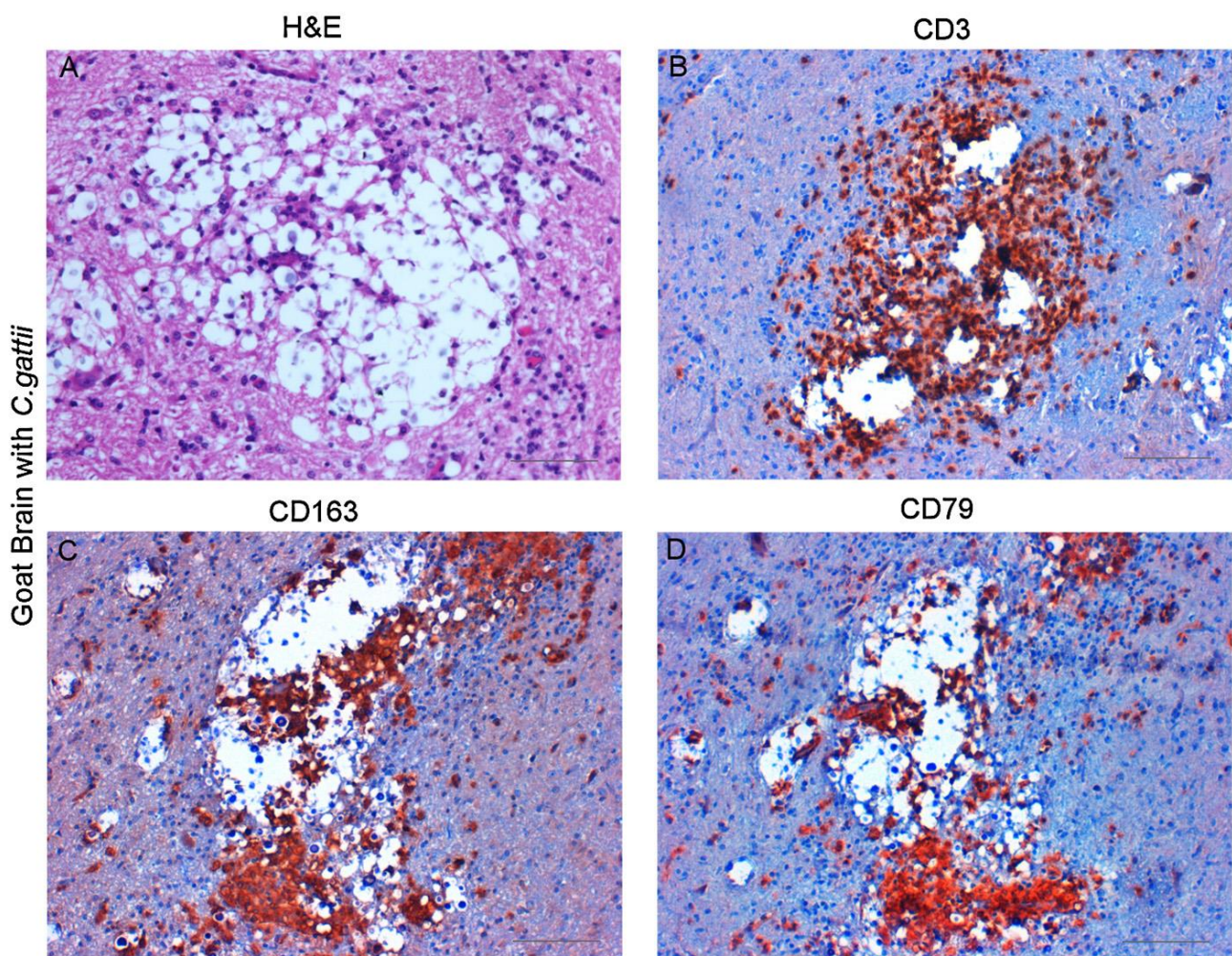


Fig. 3. In situ serotyping of *Cryptococcus* sp. affecting goat brains. (A) *C. gattii* labeled by Mab 471. (B and C) Both Mab 302 and Mab 1326 are not reactive with *C. gattii*, respectively. (D–F) Mab 471,

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.

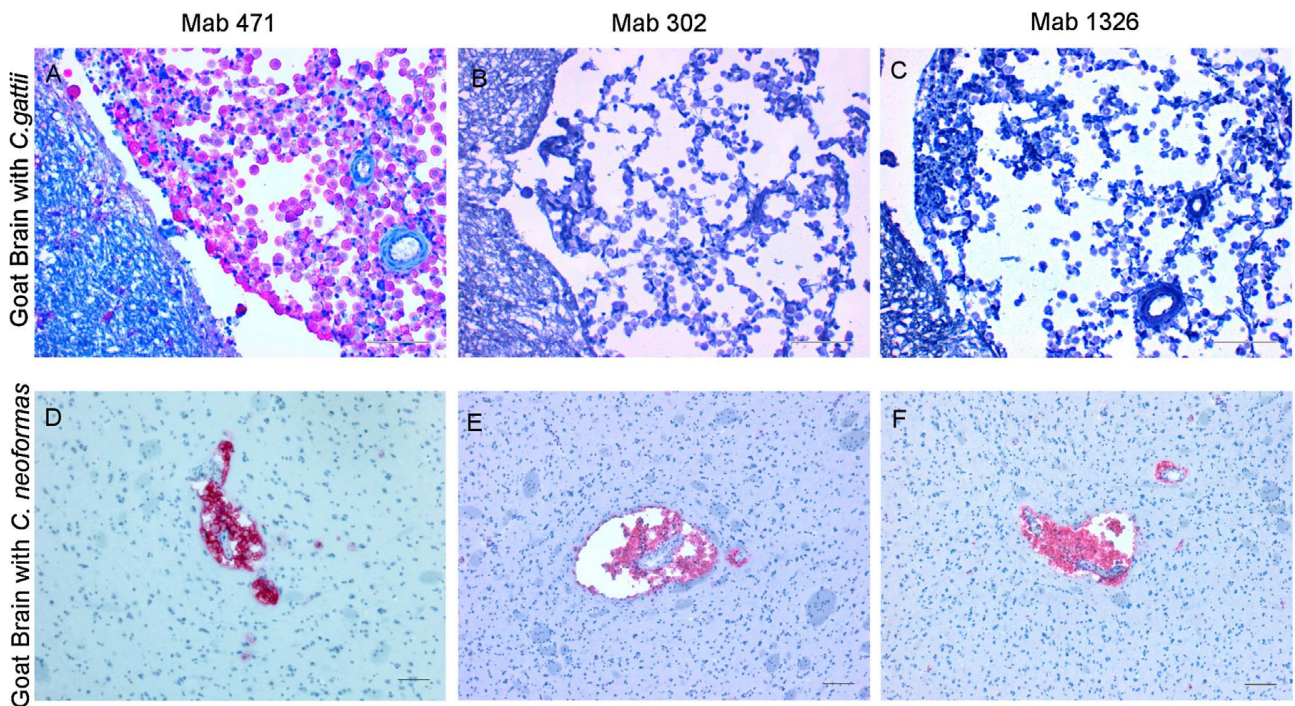


Table 1 In situ serotyping and MLST genotyping results from small ruminants with neurological cryptococcosis. Animal species Year In situ serotyping MLST genotyping Goat 1994 *C. neoformans* *C. neoformans* v. *neoformans* Goat 2003 *C. gattii* *C. gattii* Mouflon 2004 *C. neoformans* *C. neoformans*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