A large deletion in the *Plasminogen* gene is associated with ligneous membranitis in a Maltese dog

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Summary

Ligneous membranitis/conjunctivitis (LM, OMIM 217090) is a hereditary disorder caused by a congenital plasminogen (PLG) deficiency. In veterinary medicine, LM (OMIA 002020-9615) has rarely been reported in Golden Retrievers, Yorkshire Terriers, Doberman Pinschers and Scottish Terriers. In the latter breed, an A>T variation in an intron donor site of the PLG gene (PLG, c.1256+2T>A) has been found to be the sole causative molecular defect reported to date in dogs. Owing to the absence of plasmin enzymatic clearance which in turn depends on the lack of its proenzyme plasminogen, fibrin deposits tend to accumulate in viscous membranes on the eyes, triggering and sustaining an intense inflammatory response. A case of LM was diagnosed in a 7-month-old male Maltese dog. The dog was examined for severe recurrent conjunctivitis. A diagnosis of ligneous conjunctivitis was made by an ophthalmologist after a thorough eye examination and was confirmed by a complete lack of plasma activity of plasminogen. The main local signs were redness of the conjunctiva with persistent membranes having ligneous (wood-like) membranes on the eyes. The disease was associated with a complex rearrangement involving the plasminogen gene loci, causing the complete deletion of exon 1. This study provides a spontaneous animal model for LM associated with complete plasminogen deficiency and provides a method for detecting affected or carrier dogs.

Keywords animal model, complex rearrangement, deletion, dog, hereditary disease, ligneous membranitis, ophthalmology, plasminogen.

Ligneous membranitis/conjunctivitis (LM, OMIM 217090) is a hereditary disorder with a predominant recessive inheritance pattern, rarely reported in humans. It is triggered by congenital plasminogen (PLG) deficiency (Borel 1934; Bateman et al. 1986). PLG deficiency can be classified into two types: true PLG deficiency (type I or hypoplasminogenemia), associated with the primary deficiency of activity and antigen levels of plasminogen (Schuster et al. 1997; Schuster et al. 1999; Tefs et al. 2006), and dysplasminogenemia (type II), associated with decreased plasminogen activity and a normal or slightly reduced antigen level. The latter case is also characterized by almost absent clinical signs (Schuster & Seregard 2003).

In veterinary medicine, LM (OMIA 002020-9615) has been reported as a sporadic single case and as small case series in Golden Retrievers (McLean et al. 2008), Yorkshire Terriers (Torres et al. 2009), Doberman Pinschers (Ramsey et al. 1996) and Scottish Terriers (Mason et al. 2012a,b). In Doberman Pinschers and Scottish Terriers, the disease has not been strictly associated with the plasminogen deficiency. At the molecular level, to date, the only variation reported in dogs in association with LM has been an A>T variation in an intron donor site of the PLG gene (PLG, c.1256+2T>A) in Scottish terriers (Ainsworth et al. 2015).

Plasminogen is primarily synthesized by the liver tissue (Raum et al. 1980; Law et al. 2016) although, in humans, the cornea has been described as an extrahepatic site of PLG synthesis (Twining et al. 1999). In healthy individuals, plasminogen is then converted into the fibrinolytic enzyme plasmin by activators released by the cornea, which rapidly clears the cornea of fibrin deposits (Mirshahi et al. 1996). In LM, the signs are caused by the accumulation of fibrin deposits owing to the absence of plasmin enzymatic clearance which, in turn, depends on a lack of proenzyme plasminogen. The fibrin tends to accumulate in viscous membranes which tenaciously adhere to the mucusal surfaces, triggering and sustaining a strong inflammatory response.
response. The persistence of membranes and mucosal inflammation leads to the finding of ligneous (wood-like) membranes on the eyes which, together with the redness of the conjunctiva, confers the name of the disease. However, the eye is not the only organ involved as the ears, the tracheobronchial apparatus and the kidneys may also be compromised with disease progression (Marcus et al. 1990; McLean et al. 2008; Torres et al. 2009; Mason et al. 2016; Moyer et al. 2016).

In the present study, a case of LM was diagnosed in a 7-month-old male Maltese dog. The dog, weighing 5.5 kg and housed in the local dog shelter, was examined for severe recurrent conjunctivitis. A diagnosis of ligneous conjunctivitis was made by an ophthalmologist after a thorough eye examination. The main local signs were redness of the conjunctiva with persistent membranes featuring ligneous (wood-like) membranes on the eyes (Fig. S1a). Treatment for recurrent conjunctivitis was made by an ophthalmologist after a thorough eye examination. The dog was re-evaluated again 3 months later. The therapy was effective in mitigating and almost resolving the clinical signs (Fig. S1b). Furthermore, the dog was also referred to the Veterinary Teaching Hospital for a complete physical examination, involving diagnostic imaging (chest radiographs and abdominal ultrasound), complete blood count, biochemical panel coagulation testing and urinalysis, which included proteinuria measurement, and blood pressure measurement. All examinations and analyses were unremarkable with the exception of cryptorchidism and a systolic pressure of 185 mmHg measured repeatedly using a validated oscillometric technique and the appropriate cuff in the forelimb. The dog seemed uncomfortable and anxious despite numerous attempts; therefore, no definitive hypertension diagnosis could be ascertained. Although no systemic involvement was evidenced at diagnosis owing to the young age of the affected dog, the animal was re-evaluated over time to monitor the continuing ocular therapy in order to control the ocular signs. In fact, in the majority of cases, the ocular involvement tends to worsen over time. It was recently found that the transplantation of the amniotic membrane associated with immunosuppressive azathioprine induced long-term control of the ocular signs (O’Leary et al. 2018).

The dog was relatively well until it was 18 months old. At that time, more complications began to occur. The dog developed chronic coughing; radiographs showed a moderate generalized pulmonary bronchial pattern suggestive of chronic bronchopneumopathy. Furthermore, clinico-pathological findings showed persistent left-shift neutrophilia and moderate/severe proteinuria, which was already evident when it was 12 months old. Unfortunately, the dog died spontaneously when it was 30 months old. Overall, the course of disease seemed to be progressive, but somewhat benign, and more similar to the course described in affected Yorkshire Terriers than that described in Scottish Terriers (Torres et al. 2009; Mason et al. 2016).

The citrated plasma samples harvested from the diseased dog and from three phenotypically healthy control dogs underwent additional evaluation of the plasminogen activity using a chromogenic assay (Precimat, Roche Diagnostics). The plasminogen activity was not detectable in the former and was within the normal range in the latter dogs (50–60% of activity).

Assuming the complete absence of plasminogen activity to be indicative of a type I primary deficiency, a candidate gene approach giving priority to PLG gene was attempted to identify the genetic defect underlying the disease in this dog. Hence, the genomic DNA was purified from EDTA anticoagulated blood using a silica-based column method according to the manufacturer’s instructions (NucleoSpin Tissue, Macherey-Nagel).

The already known PLG, c.1256+2T>A variation was preliminarily ruled out by PCR amplification and direct Sanger sequencing. All 19 exons of the gene were then amplified by designing primer pairs (see Supporting Information) which included the entire exons and the flanking 5’ and 3’ intronic regions. All of the PCR reactions were carried out using Phusion Taq polymerase. The PCR products were checked on agarose gel for amplicon length and were compared with those of a phenotypically healthy dog. The amplicon visualization evidenced a complete lack of amplification of exon 1 only in the affected dog whereas exons 7 and 15 also gave no amplification or spurious amplicons in the control dog. Hence, the exon 1 investigation was prioritized, and additional primer pairs were designed in approximately 10 kb upstream and downstream flanking regions of exon 1, to obtain a PCR product (Fig. 1a). Finally, four ‘bridge’ PCRs (Forward 11_upstream – Reverse 2_downstream, Forward 11_upstream – Rev 1_downstream, Forward 10_upstream – Rev 2_downstream, Forward 10_upstream – Reverse 1_downstream; Supporting Information) using Long PCR Taq polymerase (Promega) were carried out using the forward primers of the PCR products successfully amplified 5’ upstream to exon 1 and the reverse primer of the first PCR product successfully amplified 3’ downstream to exon 1. The amplicon length of the ‘bridge’ PCR of the affected dog was shorter than the expected amplicon length of approximately 10 kb (Fig. 1a). The ‘bridge’ PCR amplicon was re-amplified using an inner forward primer: the amplicon was purified and sequenced using inner primers for identifying the breakpoints (Fig. 1b).

A deletion of 5986 bp (CanFam3.1 (GCA_000002285.2), Ch1g: 49,534,880–49,540,865del) involving exon 1 and the flanking regions was evidenced. The exact consequence of the deletion of exon 1 and the majority of its upstream sequences was not investigated; however, it is likely that the deletion may...
explain the complete absence of plasminogen activity found in serum. It would be interesting to carry out a mRNA expression study to assess whether mRNA expression does not occur at all or whether alternative transcripts may originate as a consequence of the deletion and then are rapidly degraded owing to a mRNA decay mechanism.

A three-primer genotyping assay assay for the rapid detection of the deletion (Gentilini et al. 2019) was established using the affected dog (Fig. 2a). To that end, genetic testing was carried out using a multiplex endpoint PCR with a mixture composed of 3 µl of 5 × PCR buffer (Phusion Green buffer), 200 µM of each dNTP, 300 nM PLG_R primer (CTTGCTTCTCATCCCTCCTATTT), 500 nM PLG_Fmut (TTCTTCACTTCCTGGGTTAAGG) primer, 300 nM PLG_Fwt (TGCAGTCCTCCTGGTTAGA), 0.3 µl/reaction of Phusion Polymerase (Thermo Scientific), 2 µl of template and molecular biology-grade water to reach a final volume of 20 µl. The PCR was carried out using a three-step protocol: initial denaturation at 98°C for 30 s followed by 40 cycles at 98°C for 10 s, 66°C for 15 s and 72°C for 15 s. The variant allele gave rise to a band 579 bp long, whereas the wt allele gave rise to a 301 bp band (Fig. 2b).

The three-primer genotyping assay was used to assess the pathogenic variant allele frequency in a sample of Maltese dogs taken from three laboratories (two from Italy and one from the Czech Republic). Among 68 Maltese dogs, no variant alleles were found. The upper 95% confidence level, estimated assuming an allele sample equal to 136 and a test sensitivity and specificity equal to 0.99, was 2.67% (https://epitools.ausvet.com.au/trueprevalence).

This study provided a spontaneous animal model for LM associated with complete plasminogen deficiency, and greatly facilitates the diagnosis of affected or carrier dogs. Despite the deletion being found in homozygosity, a limited...
epidemiological survey showed that it is currently a rare or very rare variant in the breed.

Acknowledgement

Open Access Funding provided by Universita degli Studi di Bologna within the CRUI-CARE Agreement. [Correction added on 20 May 2022, after first online publication: CRUI-CARE funding statement has been added.]

Data availability statement

The nucleotidic sequence of the allele-containing variant was submitted to GenBank and is accessible under the following submission ID: MW815970.

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Figure 2 Three primers genetic assay. (a) Schematic representation of the genetic testing assay; the common forward primer PLG_F together with two different reverse primers, PLG_Rmut aligning in the conserved region downstream of the deletion coding sequence and PLG_Rwt aligning in the 5.99 kb deleted sequence are used to amplify the locus in a multiplexed polymerase chain reaction assay. (b) A representative example of the results of the genotyping assay showing the 579 bp long and the 301 bp long bands. Lanes: A1, ladder; B1, affected; C1, carrier; D1, healthy.
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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** In bold are the primers used in the ‘bridge’ PCRs for amplifying the breakpoint region of the deletion.

**Figure S1** (a) Bilateral conjunctivitis at admission when the dog was 7 months old. (b) Three months later, after institution of therapy, the signs are resolving.

**Figure S2** Complete findings of PLG exon screening.