DOI: 10.1002/vms3.572

CASE REPORT

WILEY

Detection of aluminium hydroxide-induced granulomas in sheep by computed tomography: A feasible approach for small ruminant lentiviruses diagnosis and research

Ana Rodríguez-Largo[1,*] | Enrique Castells[2,*] | Ricardo de Miguel[1] | Álex Gómez[1] | Héctor Ruiz[1] | Marta Pérez[3,4] | Ramsés Reina[5] | Luis M. Ferrer[1,4] | Lluís Luján[1,4]

[1] Department of Animal Pathology, University of Zaragoza, Zaragoza, Spain

[2] Centro Clínico Veterinario de Zaragoza, Zaragoza, Spain

[3] Department of Anatomy, Embryology and Animal Genetics, University of Zaragoza, Zaragoza, Spain

[4] AgriFood Institute of Aragon (IA2), Zaragoza, Spain

[5] Institute of Agrobiotechnology, CSIC-Government of Navarra, Mutilva, Spain

Correspondence
Lluís Luján, Department of Animal Pathology, Veterinary Faculty, University of Zaragoza, 177 Miguel Servet Street, Zaragoza 50013, Spain.
Email: Lluis.Lujan@unizar.es

[*] Both the authors contributed equally to this work.

Funding information
Spanish Ministry of Science, Innovation and Universities, Grant/Award Number: RTI2018-096172-B-C33; Recognized Research Groups of Government of Aragón, Grant/Award Number: A17_17R

Abstract

Aluminium (Al) hydroxide use as adjuvant induces local formation of long-lasting subcutaneous granulomas in sheep. Macrophages within these granulomas have been identified as a new small ruminant lentivirus (SRLV) replication site in naturally infected animals. Diagnosis of Al hydroxide-induced granulomas in sheep is mostly based on postmortem observations but little information is available on in vivo detection. Computed tomography (CT) is used for studying these reactions in other animal species. To determine if CT could be a tool for in vivo diagnosis and research of subcutaneous Al hydroxide-induced granulomas in sheep. A retrospective survey on thoracic CT scans was performed on 46 adult sheep. Analysis included absence or presence, number and location of subcutaneous nodules. Thoracic CT scans and pathological studies were prescribed to two further sheep. Single or multiple subcutaneous nodules were detected in 26 (56.52%) sheep. One or two nodules per animal were most often observed (36.95%). Size ranged between 1.5 and 4.5 cm. Pre-contrast two-dimensional (2D) CT images showed focal or multifocal increases in subcutaneous tissue thickness. Post-contrast 2D CT images revealed hypointense areas in the centre. Histopathology indicated the presence of granulomas composed by a large number of activated macrophages, surrounding a central core of necrosis. Large intracytoplasmic Al-positive aggregates were demonstrated by lumogallion staining. CT is a useful tool to detect subcutaneous Al hydroxide-induced granulomas in vivo in sheep. CT provides a diagnostic and research tool that can be very useful in future works in Al hydroxide-induced pathology, SRLV infection, or both.

KEYWORDS
non-invasive diagnosis, ovine, small ruminant lentiviruses, subcutaneous granuloma

1 | INTRODUCTION

Aluminium (Al) hydroxide is one of the most commonly used adjuvants in sheep vaccines as it enhances a strong immune response against antigens (Bastola et al., 2017). However, injection of Al hydroxide-based vaccines can induce local formation of granulomas in several animal species such as sheep (Asín et al., 2019), cows (Marcato, 1990), cats (Graf et al., 2018), pigs (Valtulini et al., 2005), and also in humans (Gherardi et al., 2001). Granulomas are chronic inflammatory reactions triggered by a wide variety of persistent agents, characterized by the recruitment and activation of macrophages, occasionally presenting areas of central necrosis (Kumar et al., 2020). These persistent reactions induced by Al have been linked to the development of local and systemic manifestations in a variety of species. In cats, the development of injection site sarcomas has been associated to the use of Al as adjuvant (Hendrick et al., 1992) and similar reactions have been reported in ferrets and dogs (Munday et al., 2003; Vascellari et al., 2003). In sheep, these granulomas are constant and long-lasting reactions located in the subcutaneous tissue after every single inoculation of Al-containing products (Asín et al., 2019). Recently, macrophages within Al hydroxide-induced granulomas have been identified as a small ruminant lentivirus (SRLV) replication site in naturally infected sheep (Echeverría, de Miguel, Asín, et al., 2020). SRLV infection is widespread in sheep all over the world, sometimes with remarkably high individual and flock seroprevalence (de Miguel et al., 2021). This multisystemic, chronic infection causes several types of production loses and limitations to trade of live animals (Echeverría, De Miguel, De Pablo-Maiso, et al., 2020). SRLV almost exclusively replicate in activated macrophages, using cellular tools for its own replication (Blacklaws, 2012). In these circumstances, it is likely that these SRLV-infected granulomas play an important role in SRLV pathogenesis (de Miguel et al., 2021).

Computed tomography (CT) has demonstrated to be an outstanding technique in the assessment of soft tissue, allowing the acquisition of cross-sectional images that can provide detailed information on location and internal structure of subcutaneous nodules (Subhawong et al., 2010). In cats, CT has been used for the accurate diagnosis of these local injection site reactions (Travetti et al., 2013). However, diagnosis of Al hydroxide-induced granulomas in sheep is mostly based on postmortem observations (Asín et al., 2019). In sheep, this reaction is usually only detected when the necrotic content oozes to the exterior, possibly leading to an underestimation of these subcutaneous nodules. The aim of this study is to demonstrate that subcutaneous, Al hydroxide-induced granulomas are readily identifiable by CT in sheep, improving current approaches based on necropsy findings. This technique provides a useful tool for in vivo diagnosis and research in both Al hydroxide-induced pathology and SRLV infection.

2 | MATERIALS AND METHODS

This study is a retrospective survey performed on thoracic CT scans obtained from 46 Rasa Aragonesa, adult sheep, referred between 2017 and 2019 from different flocks to the Small Ruminant Clinical Service (SCRUM, University of Zaragoza). Thoracic CT images were independently reviewed by two authors, and analysis of subcutaneous granuloma-like nodules is based on (i) absence or presence, (ii) number and (iii) location.

After this retrospective study, two further sheep (sheep A and B) originating from a single flock were referred to the SCRUM due to upper respiratory tract sings. Health management plan in this flock included periodic deworming and vaccination against clostridial diseases. To establish diagnosis and possible therapeutic approaches, both animals were prescribed several clinical examinations and a complete CT study. To perform it, sheep were sedated intravenously with dexmedetomidine (5 μg/kg) combined with buprenorphine (0.01 mg/kg). General anaesthesia was achieved with propofol (1–2 mg/kg, intravenous) and maintained with isofluorane in 100% oxygen. Sheep were positioned in sternal recumbent position on the scanning table with forelimbs in extension. Thoracic CT studies were performed using 2-slice CT scanner (BRIVO CT385; General Electric Healthcare, Madrid, Spain), helical scan mode, 3 mm slice thickness, 3 mm reconstruction interval, and a pitch of 1.5:1 using the following technique: 60–80 mAs tube current, 120–140 kVp tube voltage, 228 mm field of view, and lung, soft-tissue, and bone reconstruction algorithm. Window width and level were adjusted accordingly and two-dimensional (2D) multiplanar and three-dimensional (3D) reformatting were used for optimal evaluation of the images. Intravenous iodinated contrast medium (Ominopaque, Iohexol, 300 mg iodine/ml) was administered to both sheep at 1.5–2 ml/kg, and images were reacquired approximately 30 s after contrast injection.

Sheep A and B were further referred to postmortem examination based on clinician criteria. Necropsy included an exhaustive inspection of the subcutaneous tissue. Detected granulomas were fixed in 10% formalin and routinely processed for paraffin inclusion, as previously described (Asín et al., 2019). Four micrometre sections were stained with haematoxylin–eosin (HE) for histopathological examination. Lumogallion procedure, a specific Al staining technique, was performed following a previously described protocol (Asín et al., 2019; Mirza et al., 2016). Microbiological studies of granulomas were also undertaken.

3 | RESULTS

2D CT scan images and 3D CT reconstructions were performed in the 46 sheep and revealed the presence of single or multiple subcutaneous nodules resembling granulomas in 26 (56.52%) sheep, whereas 20 (43.48%) animals did not show any nodule (Table 1). One or two nodules per animal were most often observed (36.95%) with a lower proportion of sheep showing more than five nodules (4.35%; Table 1). Using 3D reconstructions, nodules were always observed as well-defined rounded to elongated structures in the subcutaneous tissue of the flank rib area. Size ranged between 1.5 and 4.5 cm with a

TABLE 1 Range of subcutaneous thoracic flank nodules detected by three-dimensional multiplanar computed tomography reconstruction. Number of sheep in each category and percentage is indicated

Number of nodules	Number of sheep	%
0	20	43.48
1–2	17	36.95
3–4	7	15.22
≥5	2	4.35
Total	46	100

homogeneous higher signal intensity compared with the surrounding subcutaneous tissue (Figure 1a).

In all sheep, including sheep A and B, pre-contrast 2D CT scan images showed focal or multifocal increases in subcutaneous tissue thickness without obvious variation of signal intensity. The administration of intravenous contrast agent enhanced the presence of hypointense areas in the centre, an image compatible with necrosis (Figure 1b). At postmortem, sheep A and B showed a total of five and four subcutaneous nodules, respectively. Grossly, nodules were firm and showed a similar morphology and diameter to those observed by CT. On cut section, nodules exhibited a central area of necrosis delimited by a thin fibrous capsule (Figure 1b). Histopathology confirmed that nodules were granulomas composed by a large number of activated macrophages and multinucleated giant cells surrounded by small aggregates of lymphocytes enmeshed in outer bands of fibrous connective tissue. The granuloma usually surrounded a central core of necrosis. Activated macrophages contained granular intracytoplasmic material (Figure 1c). Lumogallion staining demonstrated the presence of large intracytoplasmic Al-positive aggregates within activated macrophages and multinucleated giant cells (Figure 1d). Bacteriological cultures from nodules from sheep A and B were negative.

4 | DISCUSSION

The present work demonstrates that CT is a useful tool to detect subcutaneous Al hydroxide-induced granulomas in vivo in sheep. Compatible nodules were retrospectively found in 26 out of 46 sheep, and prospectively in sheep A and B sharing the same CT characteristics either in 2D or 3D images. Moreover, pathological studies in sheep A and B verified that nodules were Al-containing granulomas. Therefore, it is possible to hypothesize that the vast majority – if not all – nodules detected by CT in the 26 sheep were Al hydroxide-induced granulomas as well. Differential diagnosis would be limited to abscesses associated with the presence of bacteria (Mauldin & Peters-Kennedy, 2015), cutaneous cysts (Goldschmidt & Goldschmidt, 2016) or tumours (Minguijón et al., 2013) but these pathologies are not frequent in the flank of sheep (L. Luján, personal observations). Histopathology confirmed the final diagnosis as granulomas, whereas bacteriological cultures revealed no growth in any sample.

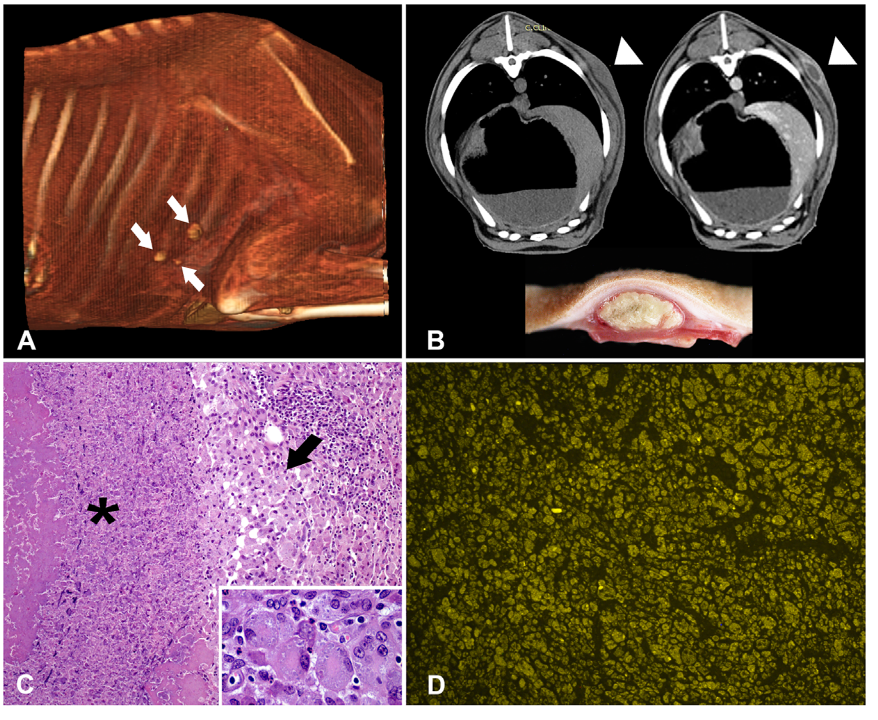

FIGURE 1 Subcutaneous soft-tissue nodules in adult sheep. (**a**) Subcutaneous soft-tissue nodules in adult sheep, 3D CT multiplanar reconstruction. Two conspicuous subcutaneous nodules of 1.86 and 1.16 cm in diameter and a smaller third nodule are located over the ribs (arrows). (**b**) Transverse, pre-contrast 2D CT image from the thorax demonstrating a single, bulging soft-tissue nodule in the subcutaneous tissue of the right flank (left arrowhead). Post-contrast 2D CT image from the same nodule displays a hypointense centre and a thin rim of peripheral enhancement, compatible with necrosis (right arrowhead). Gross section of the same nodule showing a massive central necrotic area delimited by a thin layer of viable tissue (below image). (**c**) Nodule (granuloma) is mainly composed of numerous activated macrophages (arrow) surrounding the central necrotic area (asterisk). HE, original objective 10×. Inset: Granular intracytoplasmic content within macrophages, compatible with aluminum. HE, original objective 60×. (**d**) Same granuloma than (c). Multiple intense, specific fluorescent signal is observed within macrophage cytoplasm indicating presence of aluminium aggregates in all macrophages present. Lumogallion staining, original objective 10×

Granulomas were observed in a high percentage of animals, a non-surprising fact because sheep are frequently vaccinated, in some flocks up to four vaccines every single year, the subcutaneous tissue of the thoracic flanks being the almost exclusive site for vaccine injection (Lacasta et al., 2015). Variable number (or absence) of granulomas between individuals could be first explained by the vaccination pressure of each flock. Moreover, Al hydroxide-induced granulomas are long-lasting structures that can remain at least 15 months in the subcutaneous tissue, although size reduction over time is expected (Asín et al., 2019).

Al-containing granulomas are a newly identified target tissue for SRLV that could alter viral pathogenesis and tissue tropism in infected animals (Echeverría, de Miguel, Asín, et al., 2020). Natural target organs for SRLV are difficult to sample in vivo, and therefore viral isolates are mostly obtained from postmortem samples of diseased animals. In this regard, a recent study has pointed out the benefit of biopsy spleen tissue in order to genetically characterize SRLV circulating strains, since classical approaches, based on culture of affected tissues, may underestimate the viral population as well as genotype composition (Colitti et al., 2019). If present, granulomas could provide an easy-to-reach tissue for advancing studies on SRLV isolation or genetic composition. Therefore, granuloma location needs to rely on precise techniques such as CT.

5 | CONCLUSION

This study demonstrates the usefulness of CT for accurate detection of Al hydroxide-induced, subcutaneous granulomas in sheep, providing a diagnostic and research tool that can be used in future works in Al hydroxide-induced pathology, SRLV infection, or both.

ACKNOWLEDGEMENTS

The authors are indebted to the Small Ruminant Clinical Service (SCRUM, University of Zaragoza) for their collaboration. Charo Puyó and Santiago Becerra are acknowledged for their technical help. Authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza. A. Rodríguez-Largo is a PhD student funded by the Spanish Ministry of Science, Innovation and Universities. Funded by a grant of the Spanish Ministry of Science, Innovation and Universities (RTI2018-096172-B-C33) and the Recognized Research Groups of Government of Aragón (A17_17R, Animal Health and Reproduction).

AUTHOR CONTRIBUTIONS

Ana Rodríguez-Largo: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing-original draft, and writing-review and editing. Enrique Castells: Data curation, formal analysis, resources and validation. Ricardo de Miguel: Conceptualization, formal analysis, investigation, supervision and writing-original draft. Álex Gómez: Data curation, formal analysis and methodology. Marta Pérez: Conceptualization, funding acquisition, investigation, methodology, project administration, writing-original draft, and writing-review and editing. Ramsés Reina: Conceptualization, funding acquisition, investigation, project administration, and writing-review and editing. Lluís Luján: Conceptualization, funding acquisition, investigation, project administration, supervision, validation, writing-original draft, and writing-review and editing.

FUNDING INFORMATION

Spanish Ministry of Science, Innovation and Universities; Grant Number: RTI2018-096172-B-C33; Recognized Research Groups of Government of Aragón (A17_17R, Animal Health and Reproduction).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.572.

ETHICAL STATEMENT

Animals used in this retrospective work were presented at the Small Ruminant Clinical Service for clinical studies and CT and postmortem studies were prescribed by the veterinarian in charge. Material used in this work was not primarily generated for research purposes and therefore no ethical approval was required.

ORCID

Lluís Luján https://orcid.org/0000-0002-2053-9842

REFERENCES

Asín, J., Molín, J., Pérez, M., Pinczowski, P., Gimeno, M., Navascués, N., Muniesa, A., de Blas, I., Lacasta, D., Fernández, A., de Pablo, L., Mold, M., Exley, C., de Andrés, D., Reina, R., & Luján, L. (2019). Granulomas following subcutaneous injection with aluminum adjuvant-containing products in sheep. *Veterinary Pathology*, *56*, 418–428. https://doi.org/10.1177/0300985818809142

Bastola, R., Noh, G., Keum, T., Bashyal, S., Seo, J. E., Choi, J., Oh, Y., Cho, Y. S., & Lee, S. (2017). Vaccine adjuvants: Smart components to boost the immune system. *Archives of Pharmacal Research*, *40*, 1238–1248. https://doi.org/10.1007/s12272-017-0969-z

Blacklaws, B. A. (2012). Small ruminant lentiviruses: Immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comparative Immunology, Microbiology & Infectious Diseases*, *35*, 259–269. https://doi.org/10.1016/j.cimid.2011.12.003

Colitti, B., Coradduzza, E., Puggioni, G., Capucchio, M. T., Reina, R., Bertolotti, L., & Rosati, S. (2019). A new approach for small ruminant lentivirus full genome characterization revealed the circulation of divergent strains. *PLoS One*, *14*, e0212585. https://doi.org/10.1371/journal.pone.0212585

de Miguel, R., Arrieta, M., Rodríguez-largo, A., Echeverría, I., Resendiz, R., Pérez, E., Ruiz, H., Pérez, M., de Andrés, D., Reina, R., de Blas, I., & Luján, L. (2021). Worldwide prevalence of small ruminant lentiviruses in sheep: A systematic review and meta-analysis. *Animals*, *11*, 1–21. https://doi.org/10.3390/ani11030784

Echeverría, I., de Miguel, R., Asín, J., Rodríguez-Largo, A., Fernández, A., Pérez, M., de Andrés, D., Luján, L., & Reina, R. (2020). Replication of small ruminant lentiviruses in aluminum hydroxide-induced granulomas in sheep: A potential new factor for viral dissemination. *Journal of Virology*, *95*. https://doi.org/10.1128/jvi.01859-20

Echeverría, I., de Miguel, R., De Pablo-Maiso, L., Glaria, I., Benito, A. A., De Blas, I., De Andrés, D., Luján, L., & Reina, R. (2020). Multi-platform detection of small ruminant lentivirus antibodies and provirus as biomarkers of production losses. *Frontiers in Veterinary Science*, *7*, 182. https://doi.org/10.3389/fvets.2020.00182

Gherardi, R. K., Coquet, M., Cherin, P., Belec, L., Moretto, P., Dreyfus, P. A., Pellissier, J. F., Chariot, P., & Authier, F. J. (2001). Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. *Brain*, *124*, 1821–1831. https://doi.org/10.1093/brain/124.9.1821

Goldschmidt, M. H., & Goldschmidt, K. H. (2016). Epithelial and melanocytic tumors of the skin. *Tumors in domestic animals* (pp. 135–139). John Wiley & Sons, Inc. https://doi.org/10.1002/9781119181200.ch4

Graf, R., Guscetti, F., Welle, M., Meier, D., & Pospischil, A. (2018). Feline injection site sarcomas: Data from Switzerland 2009–2014. *Journal of Comparative Pathology*, *163*, 1–5. https://doi.org/10.1016/J.JCPA.2018.06.008

Hendrick, M. J., Goldschmidt, M. H., Shofer, F. S., Wang, Y. Y., & Somlyo, A. P. (1992). Postvaccinal sarcomas in the cat: Epidemiology and electron probe microanalytical identification of aluminum. *Cancer Research*, *52*, 5391–5394.

Kumar, V., Abbas, A. K., & Aster, J. C. (2020). Inflammation and repair. *Robbins and Cotran pathologic basis of disease* (10th ed., pp. 100). Elsevier Inc.

Lacasta, D., Ferrer, L. M., Ramos, J. J., González, J. M., Ortín, A., & Fthenakis, G. C. (2015). Vaccination schedules in small ruminant farms. *Veterinary Microbiology*, *181*, 34–46. https://doi.org/10.1016/j.vetmic.2015.07.018

Marcato, P. S. (1990). *Anatomía e Histología Patológica Especial de los Mamíferos Domésticos* (2nd ed.). Interamericana-McGraw-III.

Mauldin, E. A., & Peters-Kennedy, J. (2015). Integumentary system. *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Sixth Edition* (pp. 509–736). *Elsevier Inc*. https://doi.org/10.1016/B978-0-7020-5317-7.00006-0

Minguijón, E., González, L., De las Heras, M., Gómez, N., García-Goti, M., Juste, R. A., & Moreno, B. (2013). Pathological and aetiological studies in sheep exhibiting extrathoracic metastasis of ovine pulmonary adenocarcinoma (Jaagsiekte). *Journal of Comparative Pathology*, *148*, 139–147. https://doi.org/10.1016/j.jcpa.2012.06.003

Mirza, A., King, A., Troakes, C., & Exley, C. (2016). The identification of aluminum in human brain tissue using lumogallion and fluorescence microscopy. *Journal of Alzheimer's Disease*, *54*, 1333–1338. https://doi.org/10.3233/JAD-160648

Munday, J. S., Stedman, N. L., & Richey, L. J. (2003). Histology and immunohistochemistry of seven ferret vaccination-site fibrosarcomas. *Veterinary Pathology*, *40*, 288–293. https://doi.org/10.1354/vp.40-3-288

Subhawong, T. K., Fishman, E. K., Swart, J. E., Carrino, J. A., Attar, S., & Fayad, L. M. (2010). Soft-tissue masses and masslike conditions: What does CT add to diagnosis and management? *American Journal of Roentgenology*, *194*, 1559–1567. https://doi.org/10.2214/AJR.09.3736

Travetti, O., di Giancamillo, M., Stefanello, D., Ferrari, R., Giudice, C., Grieco, V., & Saunders, J. H. (2013). Computed tomography characteristics of fibrosarcoma—A histological subtype of feline injection-site sarcoma. *Journal of Feline Medicine & Surgery*, *15*, 488–493. https://doi.org/10.1177/1098612X12472174

Valtulini, S., Macchi, C., Ballanti, P., Cherel, Y., Laval, A., Theaker, J. M., Bak, M., Ferretti, E., & Morvan, H. (2005). Aluminium hydroxide-induced granulomas in pigs. *Vaccine*, *23*, 3999–4004. https://doi.org/10.1016/j.vaccine.2004.06.058

Vascellari, M., Melchiotti, E., Bozza, M. A., & Mutinelli, F. (2003). Fibrosarcomas at presumed sites of injection in dogs: Characteristics and comparison with non-vaccination site fibrosarcomas and feline post-vaccinal fibrosarcomas. *Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine*, *50*, 286–291. https://doi.org/10.1046/j.1439-0442.2003.00544.x

How to cite this article: Rodríguez-Largo, A., Castells, E., de Miguel, R., Gómez, Á., Ruiz, H., Pérez, M., Reina, R., Ferrer, L. M., & Luján, L. (2021). Detection of aluminium hydroxide-induced granulomas in sheep by computed tomography: A feasible approach for small ruminant lentiviruses diagnosis and research. *Veterinary Medicine and Science*, *7*, 1757–1761. https://doi.org/10.1002/vms3.572