



Clinical course and pathogenicity of a *Mycobacterium avium* subsp. *paratuberculosis* isolate from scimitar oryx (*Oryx dammah*) in experimentally infected rabbits

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Abstract

Aim of the study: To evaluate the infectivity of a MAP type C of a scimitar oryx (*Oryx dammah*) - included in an international conservation list - that showed clinical signs and granulomatous enteritis associated with paratuberculosis in rabbits (*Oryctolagus cuniculus*) using molecular, bacteriological and pathological methodologies.

Area of study: The study was made in Tequisquiapan, Queretaro, CEIEPAA, FMVZ, UNAM, Mexico.

Material and methods: Three 6-week-old female New Zealand rabbits were orally infected for 3 consecutive days with 109 CFU of the MAP isolate. Blood and fecal samples were collected every 2 weeks for a total period of 28 weeks. IS900 PCR in blood and F57 real-time PCR in the feces were performed every 2 weeks and pathological analysis and bacteriological culture from tissue were made 28 weeks post infection.

Main results: MAP was detected by IS900 PCR in the blood of two of the three animals after 2 weeks of infection and again by F57 real-time PCR in the feces of the three infected rabbits. Infection with the MAP isolates in feces at 22 and 28 weeks post infection in one rabbit and isolate of vermiform appendix resulted in the development of granulomatous lesions in the three rabbits. The lesions were diffuse intermediate in one animal and multifocal in the other two rabbits.

Research highlights: Overall, these results demonstrated the infectivity of a MAP isolate from the scimitar oryx in rabbits.

Additional key words: bacteriological culture; granulomatous lesions; IS900 PCR; real-time F57 PCR; ruminants; experimental infection.

Abbreviations used: AFB (acid-fast bacilli); CFU (colony forming units); HE (hematoxylin-eosin); HPC (hexadecyl pyridinium chloride); MAP (*Mycobacterium avium* subsp. *paratuberculosis*); PBS (phosphate buffered saline); PCR (polymerase chain reaction); PCRq (quantitative real-time PCR); PTB (paratuberculosis); WBC (white blood cells); wpi (weeks post infection); ZN (Ziehl Neelsen).

Citation: Maldonado-Castro, E; Hernández-Reyes, AL; Arellano-Reynoso, B; Gutiérrez, CG; Alonso-Hearn, M; Chávez-Gris, G (2023). Clinical course and pathogenicity of a *Mycobacterium avium* subsp. *paratuberculosis* isolate from scimitar oryx (*Oryx dammah*) in experimentally infected rabbits. Spanish Journal of Agricultural Research, Volume 21, Issue 4, e05SC01. <https://doi.org/10.5424/sjar/2023214-19896>.

Supplementary material (Figs. S1, S2) accompanies the paper on SJAR's website.

Received: 12 Oct 2022. **Accepted:** 27 Sep 2023.

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Funding agencies/institutions	Project / Grant
UNAM, DGAPA-PAPIIT	Project IT201221 "Evaluation of control strategies and economic impact using the P35 immunogen in ruminants naturally infected with <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> "

Competing interests: The authors have declared that no competing interests exist.

Introduction

Paratuberculosis (PTB) is a chronic granulomatous enteritis of domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). PTB-associated clinical signs include diarrhea, weight loss, decreased milk production, and occasional premature death. MAP infection has also been linked to human intestinal inflammatory diseases, such as Crohn's disease (Whittington et al., 2019). Non-ruminant species, such as the wild rabbits (*Oryctolagus cuniculus*) can become naturally infected with MAP through the gut-associated lymphoid tissue, which results in diarrhea or pasty stools, weight loss, and granulomatous lesions mainly in the *sacculus rotundus* and vermiform appendix in the late phase of the infection (Mokresh & Butler, 1990; Beard et al., 2001a,b; Arrazuria et al., 2015, 2016). Wild rabbits can transmit MAP to domestic and wild ruminants when they feed on pastures where MAP-infected rabbits cohabit, as in the case of deer and cattle (Raizman et al., 2005; Judge et al., 2006). Mice, rats, and rabbits have been used as animal models for MAP infection (Mokresh et al., 1989; Talaat et al., 2020). However, animal models other than rabbits do not exhibit the characteristic clinical signs of PTB (Greig et al., 1999; Vaughan et al., 2005). Granulomatous lesions similar to those found in cattle have been observed in the *sacculus rotundus* and vermiform appendix of more than 60% of experimentally infected rabbits (Arrazuria et al., 2016). Additionally, MAP has also been isolated from the ileum, mesenteric lymph nodes, and feces of experimentally infected rabbits, suggesting that rabbits are a suitable animal model for replicating MAP infection (Mokresh et al., 1989; Arrazuria et al., 2016).

The mouse is the species most used to evaluate MAP infections due to MAP replication in the liver, spleen, and gut. However, experimental infections in mice are usually induced by intraperitoneal or intravenous injection of MAP (Talaat et al., 2020). Mice have a short lifespan making it more difficult to study a disease with a long chronic course. In addition, experimentally infected mice do not normally exhibit clinical signs and rarely develop PTB-associated granulomatous lesions in the gut (Roupie et al., 2012; Cooney et al., 2014; Ghosh et al., 2015).

It appears that scimitar oryx is particularly susceptible to MAP infection. Although the data are scarce, in a zoo colony in Italy ten animals were infected and six died with clinical signs of PTB (Pigoli et al., 2020). Similarly, in Mexico, ten animals from the same herd were infected and 50% of them were ill (Hernández-Reyes et al., 2022). This apparent susceptibility to PTB highlights the importance of MAP diagnosis in scimitar oryx, which is considered extinct in the wild (IUCN red list 2016: <https://www.iucnredlist.org/species/15568/50191470>). Since interspecies transmission of MAP poses a risk

to the conservation of animals in zoological parks, it is important to carry out pathogenicity studies to evaluate the infectivity of MAP strains in animals that may transmit the disease, such as rabbits. Therefore, the objective of this study was to evaluate the infectivity of a MAP field isolate from *Oryx dammah* (type C) in rabbits.

Material and methods

Ethics statement

The procedures performed on the animals were approved by the Institutional Subcommittee for the Care and Use of Experimental Animals of the National Autonomous University of Mexico (UNAM), registry SICUAE.DC-2019/1-2.

Obtaining MAP type C ORYX origin

A scimitar oryx from a zoological park in Mexico City showed clinical signs and lesions compatible with PTB at necropsy. Grossly the small intestine showed a red coloration and thickening of the mucosa and exhibited severe diffuse granulomatous enteritis by histopathology. An intestine section was homogenized using a tissue grinder Tenbroeck, decontaminated with 0.75% hexadecyl pyridinium chloride (HPC), and the macerate was cultured in Herrold's medium for 52 weeks (Ratnamohan & Spencer, 1986). Subsequently, the isolate was identified as MAP type C using a specific polymerase chain reaction (PCR) and DNA sequencing (Hernández-Reyes et al., 2022). The isolate was conserved in Luria Bertani media containing 20% sterile glycerol and 10% equine fetal serum (Jorge et al., 2005) and placed in liquid nitrogen until use. This MAP isolate was cultivated at 37 °C for five weeks in a shaking incubator (Thermo Scientific™MaxQ 4450, Germany) in Middlebrook 7H9 liquid medium™ (Difco, Detroit, MI, USA) supplemented with 2 mg/L of mycobactin J (Allied Monitor™, Fayette, MO, USA), 10% OADC (oleic acid, albumin, dextrose, and catalase) enrichment (BBL™, USA). The MAP bacteriological culture was centrifuged at $1,699 \times g$ for 10 min and resuspended in 1 mL of phosphate buffered saline (PBS). The resultant suspension was passed through a syringe with a 27 G needle and homogenized by vortexing (Labnet International Inc, USA). The bacterial suspension was stained with 1% carbol fuchsin and quantified in a Petroff Hausser chamber that was observed under a light microscope at 40x (Leica DM 1000, USA). The viability of the bacteria and the number of colony forming units (CFU) were verified by bacteriological culture in 7H9 solid medium. Aliquots containing 10^9 CFU/mL PBS were prepared and used immediately.

Experimental infection and sampling

Three 6-week-old female New Zealand rabbits were adapted to the experimental conditions for 3 weeks. On week 2, the presence of coccidia in feces was ruled out by using the Flotation and McMaster technique (Alowanou et al., 2021).

At the end of the 3 weeks of adaptation, considered as day zero and before infection, blood samples were collected from the auricular vein of the infected animals in vacutainer tubes with EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The three rabbits were orally infected with 10^9 MAP CFU for 3 consecutive days. Blood and feces samples were collected every 2 weeks for a total period of 28 weeks. At 28 weeks post infection (wpi), the rabbits were sedated with xylazine (5 mg/kg) and ketamine (35 mg/kg) and later euthanized with sodium pentobarbital (60 mg/kg) overdose injected intracardially, according to the Official Mexican regulations (DOF, 2015).

Extraction of total DNA from blood, feces, and gut tissue samples

White blood cells (WBC) were obtained from blood samples by adding 1 mL of erythrocyte lysis buffer to 500 μ L of blood samples. The tubes were vortexed and spun at $20,200 \times g$ for 2 min. The cell pellet was treated again with erythrocyte lysis buffer until it became white according to Singh et al. (2010). Subsequently, DNA was extracted from WBC using a QIAamp DNA mini-kit spin column (QIAGEN®, Duesseldorf, Germany). DNA extraction from fecal samples was performed according to Garrido et al. (2000) and DNA extraction from gut tissue according to Ratnamohan & Spencer (1986). Finally, the isolated DNAs were stored at -20°C until further use.

IS900 PCR from WBC and tissue DNA

Specific primers for the amplification of the insertion sequence IS900 of MAP, P3N (5'GGGTGTGGCGT-TTTCCTTCG3') and P5N (5'ATTCGCCGCCACCGC-CACG3') were used according to Favila-Humara et al. (2010). The PCR master mix contained 0.5 μ g of total DNA, 20 μ L of FastStart™ PCR Master (Roche, USA), and 1 μ L of each primer at a concentration of 500 nM. The PCR amplification was performed under the following conditions: 1 cycle of denaturation at 94°C for 5 min, 35 cycles at 94°C for 40 sec, 56°C for 40 sec, and 72°C for 40 sec, with a final cycle of 72°C for 5 min, using a Px2 Thermal cycler (Thermo Electron Corporation, USA). In the positive samples, the amplification of a 314 bp fragment of the IS900 MAP sequence was observed on a 1% agarose gel stained with ethidium bromide 0.5 μ g/mL (Invitrogen, USA).

Fecal real-time PCR for the amplification of the F57 map sequence

Fecal real-time PCR was performed using the Primerdesign Ltd *M. avium* subsp. *paratuberculosis* species specific F57 DNA fragment Genesig Advanced Kit® according to the manufacturer's instructions (Roche, Germany). Each PCR reaction contained 5 ng of the DNA sample, 10 μ L of master mix, 1 μ L of probes (final concentration 0.1 μ M) and 4 μ L of RNase/DNase-free water. Five μ L of RNase/DNase-free water was used as a negative control, while 5 μ L of the positive control template provided in the kit, was used for the positive control. The PCR conditions were 1 cycle of 2 min at 95°C , 50 cycles of 10 sec at 95°C , and 60 sec at 60°C , using a Genesig q16® Thermal cycler (Roche, Germany).

Identification of map in feces using Ziehl Neelsen

The identification of acid-fast bacilli (AFB) in feces was achieved by Ziehl Neelsen (ZN) staining, following the protocol described by Jorge et al. (2005).

Anatomopathological study

Samples from the duodenum, jejunum, ileum, *sacculus rotundus*, vermiform appendix, colon, rectum, mesenteric lymph nodes, liver, kidney, spleen, tonsils, and brain were collected at necropsy. Tissues were kept frozen at -20°C until processed for bacteriological culture. For the histopathological analysis, samples were fixed in 10% formalin and embedded in paraffin. Paraffin blocks were cut at 5 μ m using an ultramicrotome and slices were mounted in a slide and stained with hematoxylin-eosin (HE) and ZN. The stained sections were examined under a light microscope. The PTB-associated lesions were classified according to Maio et al. (2011) and Balseiro et al. (2019).

Bacteriological culture from tissue and feces

Only samples from *sacculus rotundus*, vermiform appendix and lymph nodes obtained during the necropsy, as well as feces, were processed for culture. Two grams of tissue were macerated by mechanical disruption, while feces were decontaminated with 40 mL of HPC at a final concentration of 0.75% and allowed to settle for 3 days. Subsequently, 6-8 drops of the suspension were taken from the layer near the sediment and inoculated in HEYMP (Herrold's medium with egg yolk and sodium pyruvate) supplemented with 2 mg/L of mycobactin J. The tubes were incubated at 37°C for 56 weeks.

Table 1. Results of the different bacteriological, microbiological and molecular diagnostic methods after the experimental infection of three rabbits with *Mycobacterium avium* subsp. *paratuberculosis* type C.

ID	PCR IS900 blood	ZN feces smear	qPCR F57 feces	PCR IS900 VA	Bacteriological culture	Clinical signs	Gross pathology	Histopathology
1	positive	positive (22, 28 wpi)	415,282 copies (22 wpi) 523,976 copies (28 wpi)	positive (28 wpi)	negative feces positive VA (28 wpi)*	negative	VA, SC, proximal ileum with mucosal thickening, edema in Peyer's patches	Multifocal granulomatous lesions in VA, SC, LN, ileum. ZN negative
2	positive	positive (22, 28 wpi)	568,841 copies (22 wpi) 684,328 copies (28 wpi)	positive (28 wpi)	negative feces positive VA (28 wpi)*	negative	SC thickened wall	Multifocal granulomatous lesions in VA, SC, LN. ZN negative.
3	negative	positive (22, 28 wpi)	1,059,344 copies (22 wpi) 1,238,914 copies (28 wpi)	positive (28 wpi)	positive feces 22*and 28 wpi** positive VA (28 wpi)**	diarrhea and weight loss (20 wpi)	VA, SC with mucosal thickening, mesenteric lymph nodes increased in size	Diffuse intermediate granulomatous lesions in VA, SC. ZN positive. Multifocal granulomatous lesions in LN. ZN negative

ID: identification; wpi: weeks post infection; ZN: Ziehl Neelsen; VA: vermiform appendix; SC: *Sacculus rotundus*; LN: lymph node; low CFU*; high CFU**.

Results and discussion

The present study demonstrated the ability of a MAP isolate from oryx to infect rabbits via the oral route. Table 1 summarizes the results of the different bacteriological, microbiological, and molecular diagnostic methods used after the experimental infection of the three rabbits with a MAP isolate from scimitar oryx. The three infected animals developed multifocal (2/3) or diffuse lesions (1/3) in the *sacculus rotundus* and vermiform appendix at 28 wpi. Gross and microscopic lesions were not observed in the liver, spleen, tonsils, or other organs of the infected animals and IS900 PCR results were negative. MAP DNA was amplified by IS900 PCR from the vermiform appendix of the three infected animals. In addition, MAP was detected by qPCR and ZN staining in the feces of all animals at 22 and 28 wpi. Furthermore, rabbit ID3 had a MAP load in feces at 22 and 28 wpi, with more than 10^6 DNA copies per gram of feces. While ID1 and ID2 rabbits did not present clinical signs, ID3 rabbit presented diarrhea 20 wpi and thereafter had a gradual loss of body weight. Rabbit ID1 showed moderate thickening of the ileum mucosa (Fig. 1A), vermiform appendix, and mesenteric lymph node. Microscopically, rabbit ID1 showed multifocal granulomas in the *sacculus rotundus* (Fig. 1B). Rabbit ID2 showed thickening only in the

vermiform appendix, and *sacculus rotundus* mucosa with multifocal granulomas composed of macrophages in the mesenteric lymph nodes (Fig. 1C) and in the absence of AFB (Fig. 1D). In rabbit ID3, mucosal thickening was observed in the vermiform appendix and *sacculus rotundus*. Histologically, both sections presented diffuse intermediate granulomatous lesions composed of macrophages and some Langhans-type multinucleated giant cells with moderate infiltration of lymphocytes at the interfollicular level in the submucosa (Fig. 1E). The infiltrate was also composed of macrophages and scarce giant cells, with abundant amounts of AFB in the vermiform appendix (Fig. 1F) and moderate amounts of AFB in *sacculus rotundus*. In the same animal, an increase in the size and edematous appearance of mesenteric lymph nodes was observed. Histologically, multifocal granulomas composed of Langhans-type multinucleated giant cells and macrophages were observed without the presence of AFB. MAP was cultured from the feces of one of the three infected rabbits (rabbit ID3) at 22 and 28 wpi (Fig. S1 [suppl]). Bacteriological cultures from the vermiform appendix of three rabbits at 28 wpi were positive. In contrast, the bacteriological cultures from the lymph nodes of three rabbits were negative despite the presence of multifocal granulomas. The association between granulomatous lesions with abundant AFB shown

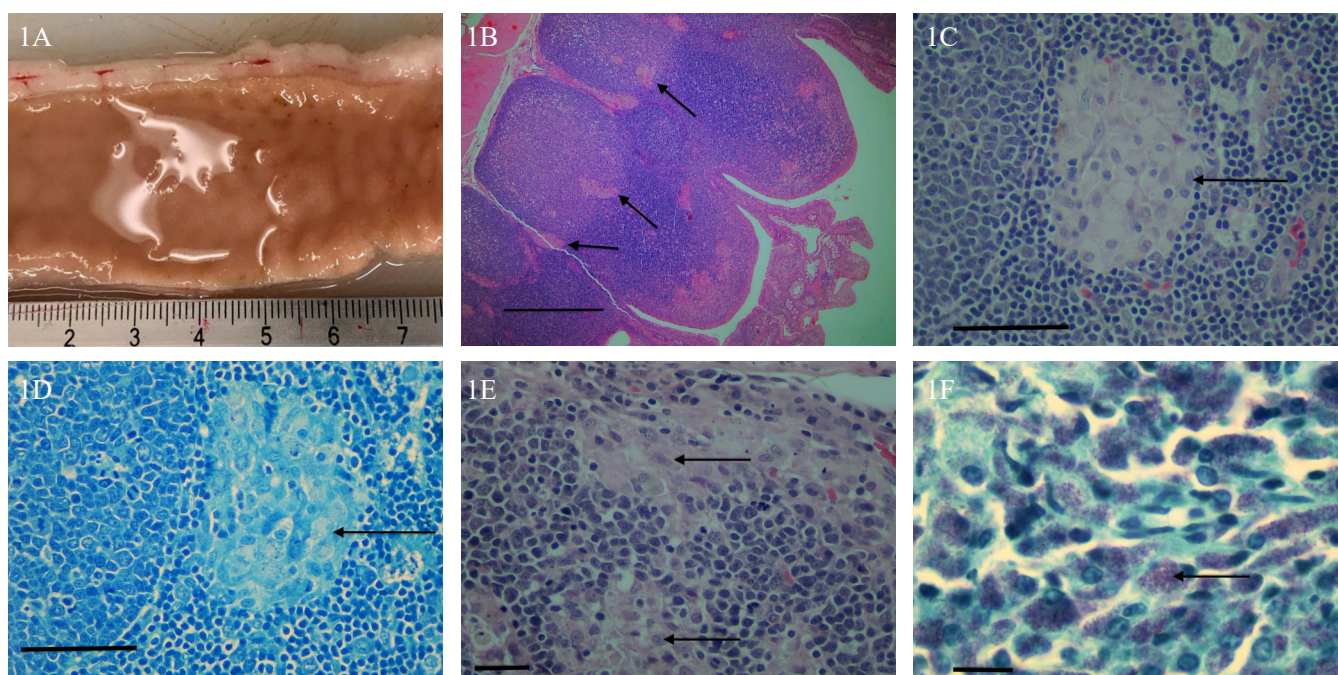


Figure 1. Results of the pathological study: (A) proximal ileum with mucosal thickening, rabbit ID1; (B) multifocal granulomatous lesions in *sacculus rotundus* rabbit ID1 (arrows); H-E 4x, bar 200 μ m. (C) multifocal granulomatous lesion in lymph node rabbit ID2 (arrow) H-E, 40x, bar 50 μ m; (D) multifocal granulomatous lesion in lymph node ZN negative, 40x, bar 50 μ m; (E) diffuse intermediate granulomatous lesions in associated lymphoid tissue in the vermiform appendix, rabbit ID3, (arrows) H-E, 40x, bar 20 μ m; (F) abundant AFB (arrow) in the cytoplasm of macrophages in lesion in the vermiform appendix, rabbit ID3, ZN positive, 100x, bar 10 μ m.

in this study and the presence of clinical signs has already been reported in both natural and experimental infections in rabbits, as well as in ruminants, where clinical signs are associated with advanced lesions, AFB and positive bacteriological cultures (Beard et al., 2001b; Maio et al., 2011). MAP was detected by IS900 PCR in the WBC of two of the three infected rabbits at two wpi (Fig. S2 [suppl]), indicative of an early systemic infection state in subclinical animals (Singh et al., 2010; Badia-Bringué et al., 2022).

Previous studies in experimental infections using rabbits have shown that not all of them develop clinical signs and lesions. These differences are due to age, breed, MAP strain type, infective dose, experiment duration, route and repeated inoculation (Mokresh et al., 1989; Mokresh & Butler, 1990; Vaughan et al., 2005; Begg & Whittington, 2008). Experimentally infected rabbits with MAP type C isolates revealed that it takes from 5 to 25 months to show clinical signs. Whereas, in some animals, lesions were observed as multifocal granulomatous with rare to moderate AFB in gut tissues and occurred between 5 to 32 months, and those without clinical signs tended to have focal lesions without AFB (Mokresh et al., 1989; Vaughan et al., 2005; Arrazuria et al., 2015, 2016). In our study, intermittent diarrhea was present in only one rabbit (ID3) at the fifth month and lesions in the intestine and lymph nodes were observed in three animals at 7 months post-infection. In experimental infection with MAP K10

isolate (type C) using the same dose as in this study (10^9 CFU), 87% of the infected rabbits developed PTB-associated lesions (Arrazuria et al., 2016). Furthermore, the different diagnostic techniques used for the evaluation of the infection may also contribute to the differences in the results between studies (Arrazuria et al., 2015, 2016). In natural conditions, it was determined that infected cattle eliminate around 10^8 CFU/g of feces, while the shedding of infected rabbits was estimated to be about $7.6 \times 10^5 \pm 5.2 \times 10^5$ CFU/g (Daniels et al., 2001, 2003). These figures for natural infection doses during extended periods of sharing pastures with infected cattle whose strains show the same genetic pattern (Greig et al., 1999) parallel the infective dose used in this study.

Conclusion

This is the first report of an experimental infection of rabbits with a MAP isolate from scimitar oryx. Our results demonstrate that rabbits can be infected with a MAP strain from oryx and develop PTB-associated lesions, mainly in the vermiform appendix and *sacculus rotundus*. We conclude that rabbits can replicate the clinical signs and the anatomical and histopathological lesions commonly observed in MAP-infected cattle. Therefore, rabbits infected with MAP are a suitable model to evaluate the pathogenesis of this disease and as vaccine candidates for the control of

PTB. In addition, our results demonstrated the infectivity of a MAP isolate from scimitar oryx in rabbits which has implications for the epidemiology of the disease. Rabbits and the scimitar oryx can be naturally infected with MAP and participate in MAP transmission, representing a risk to the conservation of animals in zoological parks where diverse animal species are closely housed.

Authors' contributions

Conceptualization: E. Maldonado-Castro, B. A. Arellano-Reynoso, C. G. Gutierrez, M. Alonso-Hearn, G. Chávez-Gris.

Data curation: B. A. Arellano-Reynoso, C. G. Gutierrez, M. Alonso-Hearn, G. Chávez-Gris.

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Investigation: E. Maldonado-Castro.

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Resources: G. Chávez-Gris.

Software: Not applicable.

Supervision: B. A. Arellano-Reynoso, C. G. Gutierrez, M. Alonso-Hearn, G. Chávez-Gris.

Validation: A. L. Hernández-Reyes.

Visualization: B. A. Arellano-Reynoso, C. G. Gutierrez, M. Alonso-Hearn, G. Chávez-Gris.

Writing – original draft: E. Maldonado-Castro.

Writing – review & editing: B. A. Arellano-Reynoso, C. G. Gutierrez, M. Alonso-Hearn, G. Chávez-Gris.

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