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Antimicrobial susceptibility testing of *Mycoplasma mycoides* subspecies mycoides isolates recovered from tissues of control and treated cattle from cattle following controlled antimicrobial treatment trials

Anne Ridley[1], Geoffrey Muuka[2], Hezron Wesonga[3], Roger Ayling[1], Jane Plater[1], Nimmo Gicheru[3], Stuke, K.4, Colston, A.[4], Salt J. [5]

[1]Animal and Plant Health Agency, Department of Bacteriology, Woodham Lane, Addlestone, Surrey, United Kingdom.
[2]Central Veterinary Research Institute (CVRI), Ministry of Fisheries and Livestock, Balmoral, Lusaka, Zambia.
[3]Veterinary Science Research Institute (VSRI), Muguga, Kenya Agricultural & Livestock Research Organization, Nairobi, Kenya.
[4]Global Alliance for Livestock Veterinary Medicines (GALVmed), Nairobi, Kenya.
[5]Global Alliance for Livestock Veterinary Medicines (GALVmed), Edinburgh, UK.
Corresponding author: info@galvlmed.org

ABSTRACT

OBJECTIVES

Two previously reported studies in Kenya and Zambia demonstrated that oxytetracycline, tulathromycin or gamithromycin treatment, following infection via an in-contact disease transmission of cattle with *Mycoplasma mycoides* subsp. *mycoides (Mmm)*, significantly reduced clinical signs, mortality, tissue lesions and spread of infection to susceptible cattle. However, with the exception of tulathromycin-treated cattle in Kenya, bacteriological clearance of *Mmm* was incomplete, with recovery of the organism from a minority of treated animals. In the present study, *Mmm* cultures from these cattle were transported to the Animal and Plant Health Agency and revived for susceptibility testing against twelve veterinary therapeutic antibiotics, including those used as treatment.

RESULTS

Following transportation and revival *Mmm* was recovered from only four of ten cultures submitted that represented antibiotic-treated animals; additionally twelve represented saline controls and untreated animals. MIC values to each of the three treatment antimicrobials were very low (≤0.125 µg/ml), with no differences between treated and untreated animals for either study. For the remaining antimicrobials, MIC values were within one doubling dilution, irrespective of experimental group. While no change to MIC value following antibiotic exposure was detected, the low number of isolates recovered from treated animals post-mortem, and subsequently available for MIC determination, prevent a meaningful assessment the effects of treatment on susceptibility of *Mmm* subsequently recovered from tissues.

Keywords

Contagious bovine pleuropneumonia (CBPP), *Mycoplasma mycoides* subsp. *mycoides*, antibiotic, susceptibility, minimum inhibitory concentration (MIC)

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a severe respiratory disease of cattle caused by *Mycoplasma mycoides* subsp. mycoides (*Mmm*). The disease is characterised by a severe fibrinous exudative pleuropneumonia, but manifests with acute or subacute symptoms, with progression to death, chronic disease or recovery. Due to its severity and high socio-economic impacts (1), (2) CBPP is listed by the Office International des Epizooties (OIE) and is an important transboundary disease, particularly affecting sub-Saharan Africa (3).

Management of the disease in endemic areas has faced substantial challenges associated with implementing quarantine and restriction of animal movement (4). Most African countries have adopted vaccination and a live attenuated T1/44 vaccine strain, originally developed in the 1960s is mostly used, conferring only short-term immunity, variable efficacy and can cause severe adverse reactions (5),(6).

Use of antimicrobials, although discouraged, is widely relied on to reduce disease impact. Clinical signs are alleviated, but concerns surrounded potential development of chronic carriers and acquired resistance to therapeutic antimicrobials (7, 8). However, there is evidence to suggest efficacy; tetracyclines prevented the spread of CBPP, despite failure to achieve bacteriological clearance in affected animals (9) and prevented formation of sequestra (10). The fluoroquinolone danofloxacin reportedly reduced transmission of *Mmm* from infected to in-contact cattle and appeared to control spread of disease and reduce deaths in naturally infected herds (2, 11-13).

With further assessment warranted, two separate experimental studies were recently carried out at the in Kenya and Zambia, using susceptible local cattle breeds, demonstrated that commercial formulations of tulathromycin and gamithromycin and tetracycline, significantly reduced clinical signs, mortality, restricted development of lung lesions and prevented transmission to animals placed in sentinel groups and co-mingled with animals within the different treatment groups (14). However, bacteriological clearance of *Mmm* was incomplete; the organism was recovered from post-mortem lung tissue of cattle treated with of tulathromycin (1/28 (4%) across both study sites) and 4/28 (14%) treated with gamithromycin. For animals treated with oxytetracycline the study reported that severe lesions were more prevalent, and *Mmm* was recovered more frequently in Zambia (6/14; 43%), suggesting that oxytetracycline may take longer, or may fail to achieve *Mmm* clearance. Reduced susceptibility to several classes of antimicrobials, including macrolides and tetracyclines, have been reported in field isolates of other pathogenic mycoplasmas of veterinary interest(15). As transmission of organisms with reduced susceptibility to antimicrobials commonly used therapeutically presents a threat to control of the disease in naïve animals, it is important to determine the susceptibility of any *Mmm* organisms subsequently recovered from body sites of antibiotic treated animals and their contacts.

In the present study, *Mmm* isolates recovered from tissue, and, where available, nasal swabs, from treated and control animals were transported for antimicrobial susceptibility to compare susceptibility to the respective therapeutic antimicrobials used as treatments.

METHODS

Origin of isolates

The experimental study from which all isolates were derived, including information on selection of animals for the study, allocation of groups, challenge, infection model, treatments, clinical and post-mortem investigations and resultant findings has been previously described by Muuka and coworkers (14), with a summary of the information pertaining to the experimental derivation of the isolates provided in Supplementary Table 1. *Mmm* cultures originally recovered and stored, from tissues (lung, pleural fluid, lymph node, nasal swabs are summarised by study treatment groups in Table 1. Where *Mmm* was successfully recovered from lung samples, no further tissue samples were microbiologically examined.

Isolates had been cultured in PPLO and confirmed as *Mmm* according to local standard operating procedures, including by PCR for selected Zambian study cultures.

Transportation and revival

Thirty six cultures representing 21 different animals included in the two studies (Table 1) were shipped to the Department of Bacteriology of the Animal and Plant Health Agency (APHA), UK on dry ice for antimicrobial susceptibility testing. In addition to those from treated animals, isolates from intubated animals, and sentinel animals, which had been used in the experiment to assess spread were also submitted.

On arrival, cultures were visually inspected and handled according to local standard operating procedures and revived in Eaton's broth medium (16). Eaton's transport broth, containing additional antibiotics, and Mycoplasma liquid medium (Mycoplasma Experience, Bletchingly, UK) was used in parallel, with subculture onto Eaton's and CBPP agars (Mycoplasma Experience, Bletchingly, UK) to aid visual identification and confirm purity. Filtration, through a 0.45µm filter (Starlabs, UK) was used where required to assist recovery. Species identification was confirmed using PCR-DGGE (17) and a confirmatory PCR (18).

MIC determination

Antimicrobials were obtained in pure form at specified concentrations on Sensititre™ plates (Thermo Fisher Scientific, East Grinstead, United Kingdom). Procedures followed those described previously (19-21), with a final test volume of 200 µl. Doubling dilutions of clindamycin, florfenicol, spectinomycin, danofloxacin, enrofloxacin, marbofloxacin, tilmicosin, tiamulin, from 0.125 µg/ml to 32 µg/ml were used, except dilutions at 4 µg/ml and 16 µg/ml were omitted. Tulathromycin and gamithromycin were provided as doubling dilutions from 0.125 µg/ml to 128 µg/ml, while oxytetracycline (0.125 to 32 µg/ml), lincomycin/spectinomycin (0.125 /0.25 µg/ml to 16/32 µg/ml) completed the plate. Two wells without antimicrobials served as growth controls.

For the MIC assay, the *Mmm* isolates were grown in Eaton's broth medium, without antimicrobials and phenol red, for 44-48 h at 37°C and adjusted to approximately 10^8 CFU/ml (20). One hundred and ninety microliters of medium was dispensed into a Sensititre™ plate followed by 10 µl of inoculum. The plates were sealed and incubated at 37°C (+/- 1.2°C) for 44 to 48h, checking after 24h. All isolates were tested in duplicate, with the tester blinded to experimental treatment group information. *Mmm* strain Afadé from the APHA culture collection was used as a control to monitor reproducibility between test batches.

Following incubation, the Sensititre™ plates were centrifuged at 800 x *g* for 3 minutes to concentrate the cells. The plates were examined with an inverted mirror in a light box to observe growth of mycoplasma. The end point was taken as the lowest dilution at which a button of cells could not be clearly visualised.

RESULTS

Nine (75%) of the 12 cultured isolates (7/10 animals) from VSRI Kenya were successfully revived and were confirmed as *Mmm* (Supplementary Table 1). These isolates represented the gamithromycin, (n=1) and saline (n=2) treated groups. The three isolates that failed to revive were originally recovered from animals in the oxytetracycline, saline and intubated groups. However, despite failing to revive, DNA prepared from an aliquot of the submitted cultures was weakly positive, by both PCR-DGGE and the confirmatory specific PCR, thus confirming the presence of Mmm DNA. *Mmm* was revived from 7/22 (32%) Zambian cultures, each from different animals; these were from the tulathromycin (n=1), oxytetracyline (n=2) and saline (n=4) treated groups. The remaining isolate was from a sentinel animal, which had been co-mingled with the saline-treated cattle. Of the fifteen submitted cultures for which *Mmm* was not recoverable, only was one positive for *Mmm* by the PCR-based tests.

MIC values for all the *Mmm* isolates against the three antimicrobials used as treatment were low, irrespective of study site and these values indicated susceptibility (Table 2). There were no differences in MIC values obtained between isolates from antibiotic-treated, and control, or intubated animals. Moreover, MIC values to the remaining eight test antimicrobials were also low (Table 2) and, for Kenyan isolates, were indistinguishable from the Afadé challenge strain. Only minor differences, within a single double dilution, were observed (Table 2), which is within the accepted natural variability of the MIC broth microdilution method.

DISCUSSION

The aim of this investigation was to identify whether treatment with commercial preparations of either gamithromycin, tulathromycin or tetracycline, in cattle experimentally infected with *Mmm* during two experimental studies conducted in Kenya and Zambia (14), was accompanied by a reduction in susceptibility to these antimicrobials.

The susceptibility of the isolates examined, as determined by MIC values to twelve different antimicrobials, recovered from antibiotic treated animals were indistinguishable to those from saline-treated animals, or untreated controls. However, as only four of the isolates available were originally recovered from animals in the antimicrobial treated groups, no statistical assessment of the impact of the antibiotic treatment on the susceptibility of *Mmm* recovered at post-mortem, which took place a minimum of 35 days after antibiotic exposure, can be made. The low MIC values obtained were not unexpected, as previous in vitro MIC studies demonstrated *Mmm* strains from different geographical origins were generally susceptible to a range of antimicrobials, as determined by low MIC values (19, 20). While not observed in the present study, reduced susceptibility to the macrolide tylosin, has been reported for some strains (20); in mycoplasmas this is usually conferred by accumulating mutations at the target site, 23S rRNA (15) and suggests reduced susceptibility associated with selective pressure attributable to common usage is possible. The reported mycoplasmacidal activity of tulathromycin against *Mmm* (22, 23) and persistence of the drug at high levels in lung tissue (24, 25) is likely to have contributed to the very low organism recovery in this treatment group, despite the reported presence of chronic and resolved lung lesions in a minority of animals (14).

As antimicrobials have been used in an unsolicited manner to treat CBPP in Africa, data aligning antibiotic use, particularly in the field, to investigations of *in vitro* susceptibility and accumulation of mutations is also required for assessing antimicrobials as a viable tool for control of CBPP. However, MIC determination for mycoplasmas and in particular *Mmm* is not widely practiced, requiring dedicated laboratory equipment and expertise. Moreoever, long-standing challenges associated with the absence of harmonised procedures, control strains and designated breakpoints for determining resistance, remain to be resolved.

Failure to recover cultures following transportation appeared to result from low numbers of viable organisms present in the submitted culture (n=3), as demonstrated by the weak confirmatory PCR test results from DNA prepared from 1ml of the submitted culture. However, from others *Mmm* was not recovered, despite use of different selective media and filtration techniques, nor was the presence of the organism confirmed by PCR-based methods. Mycoplasmas are particularly fastidious, with antibiotics normally required in culture medium to suppress contamination by faster growing bacteria, to which mycoplasma cultures are particularly susceptible. The presence of other, contaminating, bacteria competing for nutrients, was indicated for selected cultures by sequencing of unidentified bands observed after PCR-DGGE. These appeared to impede recovery of *Mmm* and highlights the challenges associated with maintaining a balance between minimising use of antimicrobials for storage of mycoplasmas prior to transportation for subsequent susceptibility testing and protecting cultures from competing, less fastidious organisms.

LIMITATIONS

- Although all *Mmm* isolates were equally susceptible to the antimicrobials under test there were insufficient numbers of isolates from *Mmm* infected, antimicrobial treated cattle, precluding meaningful assessment regarding impact of the treatments on susceptibility of *Mmm* colonising host tissues and nasal cavity.
- In large part this was due to the high metaphylactic protection afforded with the new generation macrolides used in the study, which resulted in few acute or subacute tissue lesions, with limited recovery of viable *Mmm* from tissues or nasal swabs taken at *post-mortem*.
- Additionally, some of the isolates from the different tissues from treated animals were either unavailable for transfer to the UK, or were found to be non-viable on arrival. Moreover, processing of nasal swab samples from the Kenyan study had not been completed at the time of the susceptibility testing investigations, but subsequently resulted in a further eight positive samples representing tulathromycin, gamithromycin and oxytetracycline treatment groups (14).
- While storage of isolates in media containing antibiotics is normally contra-indicated prior to antimicrobial susceptibility studies, media containing antibiotics such as penicillin (or other antibiotic classes targeting the cell wall) play an important role for mycoplasmas against contamination from competing bacteria.
- For future studies microbiological recovery and storage of *Mmm* from additional sampled tissues and pleural fluid is advisable to optimise the availability of isolates from treated animals for susceptibility investigations.

ACKNOWLEDGEMENTS

Dr Bruce Nosky (Merial Ltd) is thanked for permission to include gamithromycin on the customised Sensititre™ plates. The Bacteriology teams at the Veterinary Science Research Institute, Kenya Agricultural & Livestock Research Organisation, Muguga, Nairobi, Kenya and The Central Veterinary Research Institute, Balmoral, Lusaka, Zambia are thanked for provision of study isolates.

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ABBREVIATIONS

APHA: Animal and Plant Health Agency

BAL: Bronchio-Alveolar Lavage

BMGF: Bill & Melinda Gates Foundation

CBPP: Contagious Bovine Pleuropneumonia

CVRI: Central Veterinary Research Institute

GALVmed: Global Alliance for Livestock Veterinary Medicines

KALRO: Kenya Agricultural & Livestock Research Organisation

MIC: minimum inhibitory concentration

Mmm: Mycoplasma mycoides subspecies *mycoides*

OIE: Organization for Animal Health

PCR-DGGE: Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis

PPLO: Pleuropneumonia-like organisms

UK: United Kingdom

VSRI: Veterinary Science Research Institute

Authors' contributions

GM, HW, AC, RA developed the plan for this work. NG, BO and GM were responsible for the laboratory work at VSRI and CVRI that recovered and transferred the isolates to APHA. GM, HW, AR, RA, AC and KS contributed to the interpretation of the data and the discussion of the results. AR confirmed MIC results and wrote the manuscript. KS, GM, HW, RA, and AC edited the manuscript. All authors read and approved the submitted manuscript.

Funding statement

This work formed part of studies based on research funded in part by the Bill & Melinda Gates Foundation and with United Kingdom (UK) Aid from the UK Government through Global Alliance for Livestock Veterinary Medicines (GALVmed) (grant number OPP1009497). The findings and conclusions contained within are those of the authors and do not necessarily reflect positions or policies of the Bill & Melinda Gates Foundation or the UK Government. The funders (Bill & Melinda Gates Foundation and the UK Government) had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

The dataset analysed during the current study is available as Supplementary Table 1.

Ethics approval and consent to participate

This study further examined *Mmm* isolates from a previously described study of experimental antimicrobial treatment in cattle. This work did not involve any human experimentation. The isolates originating in Kenya were from a protocol approved by the Animal Care and Use Committee of the Veterinary Science Research Institute – Kenya Agricultural & Livestock Research Organization (approval code: KALRO-VSRI/IACUC010/07102016). Isolates from Zambia were from a protocol approved by the Zambian Ministry of Agriculture Ethics Committee (approval number 2014/001).

Consent for Publication

Not applicable. The manuscript does not contain data belonging to an individual.

Competing interests

The authors declare that they have no competing interests.

TABLES.

Table 1: Distribution of *Mmm* isolates recovered for MIC determination by experimental group

	Kenya (VSRI)			Zambia (CVRI)		
Treatment	No. (%) with *Mycoplasma* originally isolated[a]	Isolates sent (no. animals)	*Mmm* revived and confirmed by PCR-DGGE and specific PCR (no. animals)	No (%) with *Mycoplasma* originally isolated[a]	Isolates sent (no. animals)	*Mmm* revived and confirmed by PCR-DGGE and specific PCR (no. animals)
Intubation with Afadé/Caprivi strain	N/A	5 (4)	4 (4)	N/A	2 (2)	0 (2)
Saline	5/14 (36)	4 (4)	2 (4 [b])	10/13 (77)	8 (6)	4 (4)
Tulathromycin	0/15 (0)	N/A	N/A	1/13 (8)	1 (1)	1 (1)
Gamithromycin	2/15 (13)	2 (2)	1 (2)	2/13 (16)	1c (1)	0 (1)
Oxytetracycline	1/15 (7)	1(1)	0 (1 [b])	6/14 (43)	6[c](5)	2 (2)
Sentinel: in contact saline treated cattle	1/5 (20)	2(1)	2 (1)	2/5 (40)	2 (1)	0 (2 [b])

[a] Described by Muuka and colleagues(14)

[b] PCR positive but no viable Mmm recovered

[c] Includes a nasal swab culture

Table 2. MIC values for *Mycoplasma mycoides* subsp. *mycoides* isolates recovered at post-mortem from treated, sentinel and intubated cattle from both study sites

	MIC Value (µg/ml) of antimicrobial:												
	Treatment	No. isolates	OXY	TUL	GAM	TIL	TIA	DAN	ENRO	MAR	CLI	LIN	SPT	FLOR
Kenya (VSRI)	Saline	2	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25/0.5	>2 ≤ 8	0.5
	Gamithromycin	1	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25/0.5	>2 ≤ 8	0.5
	Sentinel (saline)	2*	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25/0.5	>2 ≤ 8	0.5
	Intubated	4	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25/0.5	>2 ≤ 8	0.5
Zambia (CVRI)	Saline	4	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.5	0.25	0.25	≤0.125	0.25/0.5	>2 ≤ 8	0.5
	Tulathromycin	1	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125-0.25	≤0.125-0.25	0.25	≤0.125	0.25/0.5	>2 ≤ 8	0.5
	Oxytetracycline	2	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.5	0.25	0.25	≤0.125	≤0.125/0.25 - 0.25/0.5	>2 ≤ 8	0.5

Supplementary Table 1

Study Site	Clinical Trial Group[a]	*Mmm* challenge route[a]	Antibiotic Treatment[a]	Treament dose/route[a]	Number of animals in group	Total *Mmm* positive animals/ No. animals in group at post-mortem (%)	Individual Animal Ref	Tissue Lesion type	Sample type	Viable MLO[b] recovered	Mmm PCR[c]	PCR-DGGE[d]
Kenya (VRSI)	0	Intubated Afadé	none	N/A	60	died of CBPP before end of study	K45	Not stated	Lung	+	+	*Mmm*
							K92	Not stated	Lung	-	+	*Mmm*
									Pleural fluid	+	+	*Mmm*
							K102	Not stated	Lung	+	+	*Mmm*
							K139	Not stated	Lung	+	+	*Mmm*
	1	Co-mingled with intubat-ed Group 0	Saline	6.0 mg/kg, SC	15	5/14 (36)	K14	Subacute	Lung	+	+	*Mmm*
							K58	Chronic	Lung	-	-	*Mmm*
							K149	Acute	Lung	+	+	*Mmm*
	3	Co-mingled with Group 0	Gamithromycin	6.0 mg/kg, Zactran™, SC	15	2/15 (13)	K39	Chronic	Lung	+	+	*Mmm*
	4	Co-mingled with Group 0	Oxytetracyline	20.0 mg/kg, Alamycin™ IM	15	1/15 (7)	K26	No CBPP	Lung	-	+	*Mmm*
	5	Sentinel - co-mingled with group 1[e]	none	N/A	5	1/5 (20)	K1324	Subacute	Pleural fluid	+	+	*Mmm*
									Lymph node	+	+	*Mmm*
	0	Intubated Caprivi	none	N/A	50	Completed study on day of treatment	Z131	Not stated	Tissue	-	-	Unidentified bands
							14	Not stated	Tissue	-	-	No Mycoplasma
Zambia (CVRI)	1	Co-mingled with intubated Group 0	Saline	6.0 mg/kg, SC	15	10/13 (77)	Z20	Chronic	Tissue	+	+	*Mmm*
							Z91	Chronic	Nasal swab	-	-	Unidentified bands
							Z96	Acute	Tissue	-	-	No Mycoplasma
									Nasal swab	-	-	Not done
							Z119	Acute/ Chronic	Tissue	+	+	*Mmm*
							Z129	Chronic	Tissue	+	+	*Mmm*
							Z139	Chronic	Tissue	-	-	No Mycoplasma
									Nasal swab	-	-	Unidentified bands
							Z147	Chronic	Tissue	-	-	No Mycoplasma
							Z154	Chronic	Tissue	+	+	*Mmm*
	2	Co-mingled with Group 0	Tulathromycin	2.5 mg/kg, Draxxin™, SC	15	1/13 (8)	Z117	No CBPP	Tissue	+	+	*Mmm*
	3	Co-mingled with Group 0	Gamithromycin	6.0 mg/kg, Zactran™, SC	15	2/13 (16)	Z175	No CBPP	Nasal swab	-	-	Unidentified bands
	4	Co-mingled with Group 0	Oxytetracyline	20.0 mg/kg, Alamycin™ IM	15	6/14 (43)	Z31	Acute/ Chronic	Tissue	-	-	No Mycoplasma
							Z44	No CBPP	Tissue	-	-	Not done
									Nasal swab	-	-	Unidentified bands
							Z136	Chronic	Tissue	+	+	*Mmm*
							Z143	No CBPP	Tissue	+	+	*Mmm*
							Z157	Acute	Tissue	-	-	No Mycoplasma
	5	Sentinel co-mingled with group 1[e]	None	N/A	5	2/5 (40)	Z3	Acute	Tissue	-	-	*Mmm*
							Z144	Acute	Tissue	-	-	No Mycoplasma

N/A - not applicable

a - study group as described by Muuta and colleagues (14)

b- Mycoplasma like growth and typical colonies on agar medium identified

c- PCR of Bashiruddin and colleagues, 1994

d- PCR-DGGE of McAuliffe and colleagues, 2005

e- Co-mingled with saline treated cattle

f - bands not matching known profiles for commonly identified bovine mollicutes. Sequencing of PCR products of DNA samples representing different observed profiles confirmed no match to *Mycoplasma* or other mollicutes.

g- no products detected following amplification

Doherty Building, Pentlands Science Park
Bush Loan, Edinburgh EH26 0PZ, UK

Tel **+44 (0) 131 445 6187**
info@galvmed.org

www.galvmed.org

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