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ORIGINAL ARTICLE

In vitro susceptibility testing of *Mycobacterium tuberculosis* complex strains isolated from seals to antituberculosis drugs

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Mycobacteria strains belonging to the *Mycobacterium tuberculosis* complex were isolated from seals found in the South Atlantic. The animals were received in *Mundo Marino* installations and treated for *Mycobacterium tuberculosis* complex by conventional therapy of intensive care and enriched food supply; however, in all cases treatment failed. Necropsies of all animals revealed extensive lesions compatible with tuberculosis involving lungs, liver, spleen and lymphatic nodes. Classical biochemical methods as well as molecular techniques using the IS*6110* probes were performed for mycobacterial identification. Furthermore, the LCx *M. tuberculosis* assay (Abbott Laboratories) identified all strains as *Mycobacterium tuberculosis* complex members. The *in vitro* susceptibility pattern was examined in mycobacterial strains isolated from seven seals and in 3 reference strains - BCG, H37Rv (*M. tuberculosis*) and AN5 (*Mycobacterium bovis*) - to 4 medications - isoniazid, rifampin, streptomycin and ethambutol. Minimal inhibitory drug concentrations were determined by the Mycobacterial Growth Indicator Tube (BD Argentina) method and a microdilution and colorimetric assay using 3-(4-5 dimethyltiazol-2)-2,5 diphenyltetrazolium bromide. All the isolates and the reference strains BCG and AN5 were inhibited by MIC values similar to those of H37Rv with good agreement obtained by both techniques. These findings suggest that a therapeutic regimen aimed to seals diagnosed with tuberculosis play an important role in the prevention of tuberculosis transmission from infected animals to humans that are in routine contact with them.

Key words: seals, tuberculosis, *in vitro* susceptibility testing

Susceptibilidad *in vitro* a los medicamentos anti-tuberculosos de aislados de cepas del complejo *Mycobacterium tuberculosis* obtenidos a partir de lobos marinos

Se han hallado cepas de micobacterias aisladas de lobos marinos del Atlántico sur y pertenecen al complejo de *Mycobacterium tuberculosis*. Los animales se recibieron en las instalaciones del *Oceanario Mundo Marino* y fueron tratados apropiadamente para su recuperación con la terapia convencional, cuidados intensivos y suplemento alimentario pero no se observó mejoría en su estado general. Se practicaron necropsias en todos los animales y se observaron lesiones extensas compatibles con tuberculosis en pulmones, hígado, bazo y ganglios linfáticos. Para la identificación de las micobacterias, se realizaron pruebas bioquímicas y técnicas de biología molecular con la sonda IS*6110*. Además, se identificaron todas las cepas como pertenecientes al complejo *M. tuberculosis* mediante el equipo LCx *M. tuberculosis* Assay (Abbott Laboratories). El objetivo de este estudio fue determinar *in vitro* la sensibilidad de las cepas patrón BCG, H37Rv (*M. tuberculosis*) y AN5 (*Mycobacterium bovis*) y la de las siete aisladas de lobos marinos a isoniacida, rifampicina, estreptomicina y etambutol. La concentración inhibitoria mínima (CIM) de las drogas antituberculosas se llevó a cabo con el equipo *Mycobacterial Growth Indicator Tube* (MGIT, BD, Argentina) y la microdilución con el ensayo colorimétrico con bromuro de 3-(4-5 dimetiltiazol-2)-2,5 difeniltetrazolio. Todos los aislamientos y las cepas de referencia BCG y AN5 se inhibieron con valores CIM de los de H37Rv con buena concordancia

entre los resultados obtenidos con ambas técnicas. Los hallazgos permiten sugerir que podrían ser una importante ayuda terapéutica en los lobos marinos con diagnóstico de tuberculosis y evaluar el posible papel sanitario en la prevención y transmisión de la tuberculosis de los animales a los humanos y el trabajo en conjunto.

Palabras clave: lobos marinos, tuberculosis, análisis de la sensibilidad *in vitro*

Tuberculosis (TB) continues being an important sanitary problem worldwide in human and animal health (1-3). In Argentina, TB incidence rate was of 35.5 per 100,000 inhabitants in 1997. Bovine population is estimated in almost 60 million, being 2.4 million (4.0%) of them infected with *Mycobacterium bovis*. Previous studies of genetic characterisation using IS6110 probes have demonstrated that the mycobacterial strains isolated from seals belong to the *Mycobacterium tuberculosis* complex (MTB) which consists of *Mycobacterium tuberculosis, Mycobacterium microti, Mycobacterium bovis, Mycobacterium africanum* and bacillus of Calmette and Guerin (BCG) species; more recently, three novel strains has been described: *Mycobacterium canettii, Mycobacterium caprae* and *Mycobacterium pinnipedii* sp. nov. (4-7). Seal mycobacterial isolates were found in the South Atlantic Ocean shores and they died at the Foundation of *Mundo Marino* installations (8-11). Since MTB are higly related phylogenetic species, it is reasonable to think that the antibiotics active against the rest of the species belonging to the complex, could effectively inhibit seal mycobacteria.

The aim of this study was to determine the *in vitro* activity against seal mycobacteria of the antibiotics currently used for treating TB in humans (12,13). Besides, the possible role of these drugs in a future therapeutic scheme to prevent TB transmission among animals sharing their living space and the human beings working with them, could be further evaluated (14-16).

In 1993, there was a report of 3 cases of tuberculosis in wild seals (1 New Zealand fur seal and 2 Australian sea lions) found dead on the southern coast of Western Australia. REA and hybridisation studies with oligonucleotide and RNA probes were performed to assess the similarity of strains from the wild seals to the strain previously isolated from seals at the marine park near Perth, and from the seal trainer who had workerd with the seals at the same marine park for 3 years. One of the two wild sea lion strains appeared to have identical DNA fragment patterns to the strains from the captive seal and the seal trainer. The other sea lion isolate showed minor difference with 1 endonucleases used. Differences in these isolates were more clearly seen by RFLP after hybridisation with 2 DNA probes. The wild seal isolates and *M. bovis* also differed because the wild seal isolates lacked detectable amounts of MPB 70 antigen, using a monoclonal antibody raised against MPB 70 antigen which is considered to be species specific for *M. bovis.* The secretory protein MPB 70 present in *M. bovis* was not detected in the wild seal isolates using sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting techniques. Analysis of REA and RFLP patterns indicated that the seal tuberculosis isolates were from a unique genetically distinct cluster within the *M. tuberculosis* complex.

There was a report of tuberculosis in 4 fur seals (*Arctrocephalus australis*) and 1 sea lion (*Otaria flavescens*) found dead or dying on the south western Atlantic coast of Argentina. Genomic DNA from all 5 mycobacterial isolates on Stonebrink media was analysed by the RFLP technique. Using the IS6110 probe, 4 of the isolates had identical fingerprintings with bands of 7.8, 4.2, and 1.8 kbp. The fifth isolate only had the 4.4, 2, and 1.8 kbp bands. These fingerprint patterns were different from those of *M.bovis* strain isolated from Argentinian cattle, which contained a unique 1.9 kbp band, and also differed from those human *M. tuberculosis* isolates which have many IS6110 copies.

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Materials and methods

Isolates

Seven mycobacteria isolated from seals that were found in the South Atlantic shores

Reference strains: *M. tuberculosis* H37Rv (ATCC 27294), *M. bovis* BCG (Pasteur strain), and *M. bovis* AN5.

All the mycobacteria were isolated in Stonebrink solid medium (17,18) and they were identified as part of the MTB by both biochemical and molecular techniques (17-19). In addition, LCx *M. tuberculosis* LCx a molecular, commercial and automated test (20), was used for strain identification.

Reagents

A solution of 5 mg/ml in water of 3-4 (4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), purchased from Sigma, was prepared and, then, sterilised by Millipore 22 nm filters.

A solution of 20% Tween 80 (Sigma) in water was prepared and later sterilised by autoclaving 20 min at 1 atmosphere and 121°C (14,21).

All the mycobacteria were isolated in Stonebrink solid medium (17,18) and they were identified as part of the *M. tuberculosis* complex by both biochemical and molecular techniques (17-19).

Middlebrook 7H9 liquid medium (M7H9, BD Argentina) supplemented by 10% OADC enrichment (BD, Argentina) was used to prepare the different bacterial solutions to be later inoculated in the microdilution assay.

Mycobacteria growth indicator tubes (MGIT, BD Argentina) supplemented by 10% OADC enrichment were used to determine MIC values by the macrodilution system.

Drug solutions

Antibiotics used for MIC determinations were: isoniazid (INH), streptomycin (SM), ethambutol (EMB), and rifampin (RMP). The drugs were purchased from Sigma (USA).

Stock solutions of each drug were made in water for INH, SM, and EMB and dimethylformamide (Sigma) for RMP. Final working dilutions of each drug were then prepared in M7H9.

Mycobacteria inocula

Mycobacterial suspension (S1) was prepared from a pure culture in M7H9 liquid medium supplemented by OADC enrichment. Suspension A was later adjusted for turbidity to the No. 1 McFarland standard (~4 x 10^6-10^7 CFU/ml).

Mycobacteria working suspensions S2 and S3 to be used in the MTT method were later prepared by diluting S1, 1:25 (S2), 1:2,500 (S3, 1:100 from S2) and 1:50 (S4) with M7H9.

Minimal inhibitory concentrations

The MIC of each drug was defined as the lowest concentration that completely inhibited the *in vitro* mycobacterial growth. Colorimetric microdilution assay with MTT was used for MIC determinations. Susceptibility testing of the isolates was made by macrodilution method on MGIT system .Table 1 shows the concentration range of each drug tested in this study by both of the systems used.

Determinations of colorimetric MIC

MTT assays were performed in 96 flat-bottom microtiter plates (Falcon 3072, BD, Argentina); 200 µl of sterile deionized water was added to all outer-perimeter wells to minimise evaporation of culture medium during the incubation period. The wells in row B to G in column 2 to 11 received 100 µl of M7H9 broth. One hundred µl of a solution containing INH 4.00 µg/ml was added to wells 2B and 8B. A solution of 8 µg/ml of RMP was added to wells 3B and 9B. Solutions containing 32 µg/ml of SM and 128 ug/ml of EMB were put in wells 4B and 10B and 5B and 11 B, respectively.

Wells 6 and 7 C, D, and E were used as growth control free of drugs, while wells 6 and 7 F were used as diluted 1:100 growth control (GC) also without drugs. Wells 6 B and 7 B were used as medium control containing only 200 µl of M7H9. This scheme was designed for both making the tests and later obtaining duplicate results as well as to study 2 different strains in the same assay (see scheme 1) (22).

One hundred µl of S2 bacterial suspension were added to the wells containing the appropriate dilution of each drug and control. A volume of 100 µl of the bacterial suspension S3 was put into the

well 7 F used as diluted GC. The plates, placed in plastic sealed bags were incubated at 37°C for 5 days and afterwards a mixture of 12 µl of Tween 80 and 10 µl of MTT prepared solutions were added to one of the wells used as undiluted GC. The plates were then incubated 24 hours more. If the change of colour from yellow to dark blue or purple was observed indicating mycobacterial growth, the mix of Tween 80 and MTT was added to the rest of the wells. After 24 hours of incubation under the above-mentioned conditions, visual readings were made and MIC were recorded. The concentration of drug in the well showing less change of colour than that of the 1:100 diluted GC well, was considered to be the MIC value for the assessed drug (23).

Macrodilution method of MGIT

Macrodilution MIC determination was performed using the MGIT (supplemented by 10% OADC enrichment) containing different drug concentrations (table 1) and one free of them to be used as GC. A volume of 0.5 ml of S4 bacterial suspension was added to each MGIT to a final volume of 5.0 ml. The tubes were later incubated at 37°C for a total period of 15 days and daily examined for fluorescence at 365 nm using a UV transilluminator. The time in which the growth control became positive was recorded. The drug concentration in the MGIT in which no fluorescence could be detected after the incubation time was considered to be the MIC value for the tested drug. The incubation of the MGIT was prolonged for 30 days if the tubes were negative.

Results

All the studied strains were identified as part of MTB by the LCx *M. tuberculosis* assay.

As it was considered before, MIC was the lowest concentration of each drug which inhibited more than 99% of the original population growing in a drug-free medium well (13). Table 2 shows the isolates and the reference strains BCG and AN5, inhibited by MIC values similar to those obtained for H37Rv strain. Mean value of MICs obtained by A-MTT were INH, 0.17 µg/ml (range: <0.03 µg/ml to 0.25 µg/ml); SM, 1.7µg/ml (range: 0.80-4.00 µg/ml); RMP, 0.11µg/m (range: 0.10-0.50 µg/ml), EMB, 6.00 µg/ml (range: >16.00- <2.00 µg/ml).

Good agreement among MIC values results from the tested drugs obtained by both techniques was also observed for each one of the isolates and the reference strains (tables 1 and 2). MICs of EMB showed bigger values than those found for the rest of the drugs. Significant differences in MICs by A-MTT and MGIT were observed for EMB in the strain 1864. Nevertheless, MICs by A-MTT and MGIT system were highly similar.

According to the results obtained for H37Rv, BCG and AN5 reference strains with both methods, we observed that the wild seal mycobacteria isolates were susceptible *in vitro* to the antimicrobial agents currently used in TB chemotherapy.

The microtiter system allowed determining the MIC of the drugs at a very low price (almost US $4.00 for 2 tested strains) since the microtiter plate price, which is the most expensive element in this assay, is almost US $3.00 in our country.

Discussion

Both systems used in this study for *in vitro* MIC determination, MGIT, which is a commercial macrodilution system, and MTT the in-house microdilution assay, contain the same culture liquid

Table 1. Concentration of the tested drugs used for MIC determination by the colorimetric MTT assay and the MGIT system.

Drug	MTT (µg/ml)	MGIT (µg/ml)
Isoniazid (INH)	1.00-0.50-0.25-0.13-0.06-0.03	0.10-0.50
Streptomycin (SM)	8.00-4.00-2.00-1.00-0.50-0.25	0.80-2.00-4.00-8.00
Rifampin (RMP)	2.00-1.00-0.50-0.25-0.13-0.06	1.00-0.50
Ethambutol (EMB)	32.00-16.00-8.00-4.00-2.00-1.00	8.00-4.00-2.00

MTT: 3-(4-5 dimethyltiazol-2)-2,5 diphenyltetrazolium bromide
MGIT: mycobacterial growth indicator tube

Table 2. MIC results obtained by the colorimetric MTT assay and the macrodilution MGIT system.

	MTT MIC (µg/ml)				MIC MGIT (µg/ml)			
Isolate identification	**INH**	**SM**	**RMP**	**EMB**	**INH**	**SM**	**RMP**	**EMB**
1850	0.25	2.00	<0.06	8.00	0.25	2.00	0.10	8.00
1849	0.03	2.00	<0.06	<1.00	0.10	4.00	0.10	2.00
1868	0.25	2.00	<0.06	8.00	0.25	4.00	0.10	8.00
1864	0.06	0.50	0.13	<1.00	0.25	4.00	0.00	16.00
1337	0.25	2.00	0.25	2.00	0.25	2.00	1.00	2.00
2186	<0.03	0.50	0.13	<1.00	0.10	0.80	0.50	2.00
2192	<0.03	0.50	0.13	<1.00	0.10	0.80	0.50	2.00
H37Rv	0.13	1.00	<0.06	<1.00	0.10	0.80	0.50	2.00
BCG	0.13	2.00	<0.06	2.00	0.25	2.00	0.10	4.00
AN5	0.25	4.00	0.13	2.00	0.25	2.00	0.10	4.00

medium albeit they use different final volumes and growth indicators. They were useful tools for mycobacteria detection from pure cultures after a short incubation period. Consequently, they appeared to be suitable for determining *in vitro* MIC of several antituberculosis drugs on the seal mycobacteria isolates. While both of the techniques were easy to set up in a clinical laboratory, MGIT system was safer than the microdilution MTT method. Albeit reagents used for MTT method are cheaper than those for MGIT system, the first one is much more laborious than the last one. In addition, all the tested SB strains were identified as members of *M. tuberculosis* complex by the commercial LCx assay. These facts are very important taking into account that the affected animals have a commercial value and also they are frequently living in special settings, sharing their space with human trainers (24,25). Transmission of seal mycobacteria organisms is unknown, but it could be possible that people might become infected by the airborne via as well with these mycobacteria.

In vitro activity of the antibiotics currently used in human TB chemotherapy against mycobacteria isolated from seals was observed in this study. It has suggested us the possibility to set up specific therapeutic schemes intended to improve the animal health and also to diminish the transmission of seal mycobacteria among the members of the community, as well as to the personnel involved in their care. Additional efforts should be made attempting to achieve the disease diagnosis and the seal mycobactera isolation from the live seals (13). Animals with clinical signs and symptoms of the disease (like weight loss, *nova* clinical symptom, abnormal behaviour) should be properly examined in order to confirm the diagnosis of TB. Biopsies, mouth swabs, blood and exudates from different lesions might be investigated looking for mycobacteria by performing microscopy direct examination and cultures. Today no antibiotic treatment has been tried in sea mammals, so when the decision to start has been made, periodic controls must be performed on the animals in order to evaluate the outcomes related to the proposals for these particular cases. Seals with TB diagnosis could be handled according to the currently used chemotherapy rules in human beings in order to obtain preliminary results, which could indicate needed changes to be made.

Briefly, the proposed therapy consists in the following phases:

- Two months long attack phase with 4 drugs: INH at doses of 5 to 8 mg/kg dayly; RMP at 10 mg/kg dayly; SM at 15 to 12 mg/kg dayly, and EMB at 15 to 25 mg/kg dayly.
- Four months long consolidation phase with 2 drugs: H and RMP at the above mentioned doses.

All the antibiotics except SM, which has to be injected, are administered by oral route.

Periodically, assessment of liver function and side effects of the drugs administered to the animals should be made. Furthermore, conclusions about treatment length, doses and drug composition of

the proposed regimens could be drawn out from these observations.

The assistance to stranded sea mammals not recovered due to an inadequate treatment lacking tuberculostatic drugs will be achieved by TB chemoprophilaxis.

New species typing by molecular biology probe IS6110 and LCx *M. tuberculosis* assay (Abbott Laboratories) as belonging to the *M. tuberculosis* complex.

In vitro susceptibility has been observed towards isoniazid, rifampicin, streptomycin, and ethambutol by microdilution and colorimetric assay with growth inhibition of all strains.

A sanitary role, this finding implies using the results to prevent transmission on TB in humans exposed due to their work activities: parks, oceanariums, animal trainers veterinarians, cleaning personnel and facilities maintenance staff personnel that daily feed sea mammals populations. The subspecies mentioned in this work have pathogen characteristics since they produce lesions spread in that animal that cause mortality. All this implies a sanitary problem due to the number of hours that trainers are in contact with animals and in shows to the possibility of a job-related disease, spread the disease among in-contact animals and treatment to prevent a future problem of zoonotic transmission.

Conclusion

MGIT and A-MTT microdilution techniques were useful tools for determining *in vitro* MICs of several antituberculosis drugs on seal mycobacteria isolates and, therefore, they were *in vitro* inhibited by them. These findings allowed us to suggest that it could be helpful to assess a therapeutic system aimed to treat seals with TB diagnosis. The possible treatment should be based on that for human beings regarding doses and administration principles.

In the near future, it could be helpful to develop a research project inoculating laboratory animals in order to have experimental infections, which would be further treated with antibiotic schemes including the studied drugs.

Demonstration of the ethiological agent of the disease and the possibility to start, in a short time, the adequate therapy, should be the weapons for fighting against lives and economic losses caused by this particular kind of TB.

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