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THERMORESISTANCE OF MYCOBACTERIA

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Abstract

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An investigation was made into the thermoresistance of some pathogenic species of mycobacteria in water and in liquid serum medium. A total of 105 strains of 7 species of mycobacteria *(Mycobacterium bovis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium gordonae, Mycobacterium kansasii, Mycobacterium smegmatis, Mycobacterium phlei)* were examined using a method of glass capillary tubes. They were exposed to 60 °C, 65 °C, 70 °C and 75 °C for 10, 20 and 40 seconds, 2, 4, 8, 16, 30 and 60 minutes and 2, 4, 6 and 8 hours. The lowest thermoresistance was shown by *M. bovis* strains: they were devitalized by exposure to 60 °C for as few as 16 minutes and by exposure to 70 °C and 75 °C within 10 seconds. The highest thermoresistance was shown by *M. phlei* strains: they were devitalized by exposure to 75 °C for 20 seconds.

With a simple and easily reproducible tube method using liquid serum medium for the cultivation of mycobacteria marked differences were found in the thermoresistance of the strains of *M. avium—intracellulare* complex upon their exposure to 60 °C for 2 hours in correlation with their virulence in bioassays on pullets. The virulent strain (*M. avium*, serovar 2, 3) proved less thermoresistant than the avirulent strains. The results correlated with the evaluation of growth the *M. avium —intracellulare* complex strains at different temperatures.

It is concluded that the method described makes it possible to extend the knowledge of the biological properties of individual mycobacterial species.

Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium bovis, Mycobacterium kansasii, Mycobacterium smegmatis, Mycobacterium phlei, Mycobacterium gordonae, thermoresistance

Current methods for the identification of mycobacterial species (both slow and rapid growers) include, besides a number of biochemical and other techniques, the assessment of growth at various temperatures. The growth of slow-growing mycobacteria on culture media is generally evaluated at 22 °C, 25 °C, 42 °C and 45 °C. In rapid growers the culture media are incubated up to 52 °C in view of the thermophilic properties of *Mycobacterium phlei* (Bergey 1974, 1985).

In some laboratories slow- and rapid-growing mycobacteria were differentiated using 33 °C and 39 °C in addition to the afore-mentioned temperatures (Cowan 1974). These experiences have also been adopted in standard laboratory methods for diagnosis of tuberculosis and mycobacteriosis (Kubín 1975). The possibility of using mesophilic, psychrophilic and thermophilic characteristics in the differentiation of mycobacteria is limited to only some species of slow- and rapid-growing mycobacteria. This approach as well as biochemical tests are unable to differentiate the most common pathogenic mycobacteria in animals, particularly the complex of *M. avium* and *M. intracellulare*.

In some cases, particularly in *M. avium* strains, not even serotyping can be used because of the unsuitable form of their growth (Schaefer 1965).

In the present study we attempted to find whether mycobacterial species could be identified on the basis of their different thermoresistance as is the case with some other bacteria.

Materials and Methods

For assessment of the thermoresistance of the isolated mycobacterial strains a method of glass capillary tubes 0.6 to 0.8 mm in diameter (wall thickness 0.05 to 0.1 mm) and 100 mm in length was used. Homogenous suspension of mycobacteria to be tested was drawn into a sterile capillary tube, held aslant, up to the height of about 50 mm by spontaneous capillarity. Then the capillary tube was sealed at both ends by means of a gas burner. Before the mycobacterial suspension was drawn in, one end of the capillary tube was bent so that the tube could be hung on the loop of a circular carrier prepared from a piece of wire. Before being immersed into water of a given temperature the free end of the capillary tube was weight by winding onto it a piece of thin copper wire. For identification, the capillary tubes were hung on the wire carrier in the given order and immersed into water bath of the respective temperature.

With the glass capillary tube technique as described above thermoresistance of 105 strains of 7 mycobacterial species *(Mycobacterium bovis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium gordonae, Mycobacterium kansasii, Mycobacterium smegmatis, Mycobacterium phlei)* was assessed. From the strains grown on solid egg media 2-ml homogenous suspensions were prepared in the concentration of 1 mg semi-wet bacterial culture per ml sterile distilled water. All strains were exposed to 60 °C, 65 °C, 70 °C and 75 °C for 10, 20 and 40 seconds, 2, 4, 8, 16, 30 and 60 minutes and for 2, 4, 6, and 8 hours.

After the exposure the capillary tubes hanging on the circular wire carrier were transferred to water chilled to 5 °C and then removed from the carrier one by one. In doing so, one end of each capillary tube was dried with cellulose cotton-wool, flamed and cut off with sterile shears. By heating the closed end of the capillary tube its contents were forced out directly onto solid medium and into liquid serum medium for the cultivation of mycobacteria. The final result of cultivation was evaluated after two-month incubation of the nutrient media in an incubator.

Of atypical mycobacteria the following collection strains were tested:

M. kansasii	F 29 No.	14471 ATCC	Brno
M. kansasii	F 34	P 16 Runyon USA	Brno
M. kansasii	F 35	P 8 Runyon USA	Brno
M. kansasii	F 36	P 24 Runyon USA	Brno
M. kansasii	F 49	J. Tonge, Australia	Brno
M. kansasii	F 38	Valenti, Italy	Brno
M. intracellulare	7149	Kowai 3	Brno
M. intracellulare	7151	Kowacs	Brno
M. intracellulare	7152		Prague
M. intracellulare	7153		Prague
M. intracellulare	P 25		Prague
M. intracellulare		Juhlin Enback, Copenhagen	Brno
M. intracellulare	3549	ATCC	Prague
M. intracellulare	904		Prague
M. intracellulare	P 3		Prague
M. gordonae	7179	SN 601 Borstel	Brno
M. gordonae	7180	SN 632 Borstel	Brno
M. gordonae	7181	SN 645 Borstel	Brno
M. gordonae	7182	SN 651 Borstel	Brno
M. gordonae	7183	SN 703 Borstel	Brno
M. gordonae	301 D		Prague
M. gordonae	120 D		Prague
M. gordonae	163 D		Prague
M. gordonae	285 D		Prague
M. gordonae	S 18		Prague
M. smegmatis	7200	SN 2 Borstel	Brno
M. smegmatis	7201	SN 10 Borstel	Brno
M. smegmatis	S I	VÚVeL	Brno
M. smegmatis	S 00	VÚVeL	Brno
M. smegmatis	Rb I	VÚVeL	Brno
M. smegmatis	Rb II	VÚVeL	Brno
M. phlei	3553	ATCC	Prague
M. phlei	7204	SN 101 Borstel	Brno
M. phlei	7205	SN 104 Borstel	Brno
M. phlei	7206	SN 105 Borstel	Brno
M. phlei	7207	SN 110 Borstel	Brno
M. phlei	7208	SN 112 Borstel	Brno

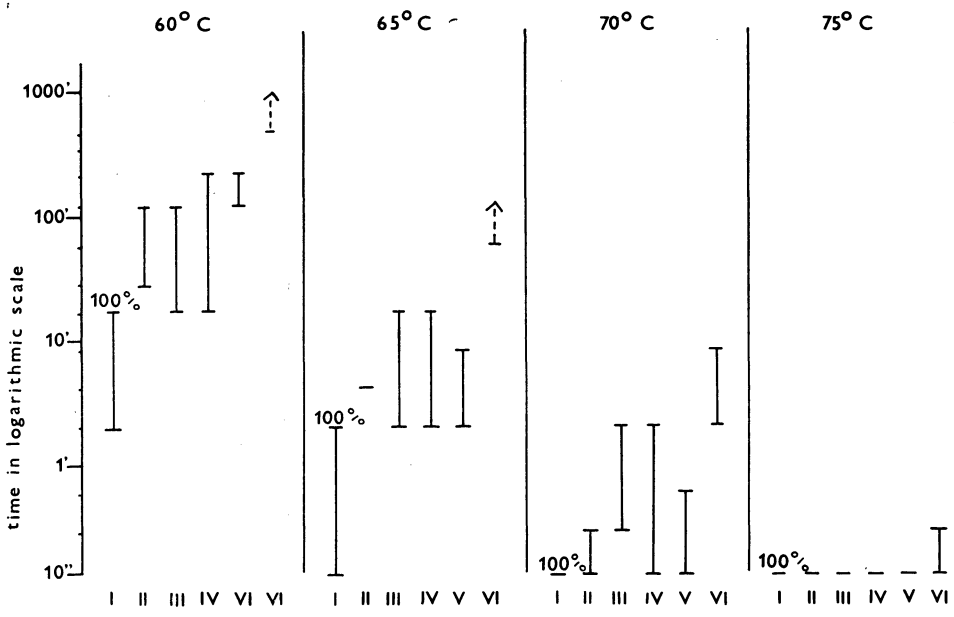

Fig. 1. Death rate of mycobacteria in water

I *M. bovis*	IV *M. avium—intracellulare*
II *M. kansasii*	V *M. smegmatis*
III *M. gordonae*	VI *M. phlei*

The strains of *M. bovis* and *M. avium* tested in the present study were isolated by us from lymph node and organ samples obtained from tuberculous pigs, cattle and domestic fowls in the course of several years.

In view of the results obtained upon exposure to 60 °C for 2 to 8 hours a procedure simpler than the capillary tube technique was adopted in the next phase of our experiments. With this modified method the thermoresistance of mycobacteria was tested in liquid serum medium for cultivation of mycobacteria (Institute of Sera and Vaccines, Prague) heated to 60 °C for the given lengths of time immediately after inoculation. The were media inoculated with 0.5 ml of 4- to 6-week culture of mycobacteria grown in serum medium. After cultivation the tubes were immersed in water bath of 60 °C (± 1 °C) for 30 minutes and 1, 2 and 4 hours. Afterwards they were cooled by immersion into cold water and placed in an incubator where they were kept at 37 °C. The resultant growth was evaluated after 1 and 2 months. The results of thermoresistance were checked by assessing the virulence of the mycobacterial suspensions by intramuscular inoculation of pullets in the dose of 1 mg culture/1 ml saline. For these experiments we used a total of 141 *M. avium — M. intracellulare* strains isolated from lymph nodes and organs of pigs and domestic fowls; 82 of them proved avirulent and 59 produced generalized organ tuberculosis in expeimental pullets. Most of the strains avirulent for pullets were classified within serovar A 8. The virulent strains producing generalized organ tuberculosis in experimental birds were classified mostly within serovar A 2, 3 and occasionally within serovar A 1 on the basis of rapid and slow agglutination (Schaefer 1965).

Results

The results of testing the individual mycobacterial species for thermoresistance by exposure to 60 °C, 65 °C, 70 °C and 75 °C for 10 seconds to 4 hours by means of the glass capillary tube technique are summarized in Tables 1 and 2 and in Fig. 1. The least thermoresistance was shown by *M. bovis* strains: they were

Table 1

Thermoresistance of mycobacteria at 60 °C

Mycobacterium species	No. of strains	Devitalization of the strains (in %) exposed to 60 -C for												
		seconds			minutes						hours			
		10	20	40	2	4	8	16	30	60	2	4	6	8
M. bovis	38	0	0	0	29,4	74	88,5	100	100					
M. kansasii	6			0		0		0	66.7	83.3	100			
M. gordonae	10				0	0	0	20	36.4	90.9	100	100		
M. avium — intracellulare	39						0	2.5	20.5	23	43.5	100	100	
M. smegmatis	6							0	0	0	77.8	100	100	100
M. phlei	6							0	0	0	0	0	0	33.3

Table 2

Thermoresistance of mycobacteria at 65 °C, 70 °C and 75 °C

Mycobasterium species	No. of strains	Devitalization of the strains (in %) exposed to 65 °C, 70 °C, and 75 °C																	
										70 °C						75 °C			
		seconds			minutes					seconds			minutes			seconds			
		10	20	40	2	4	8	16	30	10	20	40	2	4	8	10	20	40	
M. bovis	38	40.5	75	98	100	100	100			100	100	100				100	100		
M. kansasii	6		0	0	0	100	100	100		73.7	100	100	100			100	100	100	
M. gordonae	10		0	0	28.5	63.6	90.9	100		0	54.5	75	100	100		100	100		
M. avium — intracellulare	39		0	0	20.5	38.4	84.6	100		35.9	64.1	94.8	100			100	100		
M. smegmatis	6		0	0	37.5	80	100	100	100	28.5	71.5	100	100	100		100	100		
M. phlei	6				0	0	0	0	0	0	0	0	36.4	50		90.9	100	100	

devitalized at 60 °C within 16 minutes, at 65 °C in 2 minutes and at 70 °C and 75 °C within 10 seconds. The highest thermoresistance was shown by *M. phlei* strains: they were devitalized at 75 °C in 20 seconds.

Considering that the greatest differences in thermoresistance between the individual mycobacterial species were found upon exposure to 60 °C for 2 to 8 hours the next phase of our experiments was carried out using the simple and easily reproducible tube method. With this technique we found marked differences in thermoresistance in the *M. avium — M. intracellulare* complex between strains virulent and avirulent for pullets. Of 59 strains that proved virulent in bioassay on pullets only 2, i.e. 3.4 %, grew in liquid serum medium heated to 60 °C after 2 hours (Table 3). These results are in correlation with the growth of mycobacteria in liquid serum medium at different temperatures where virulent cultures of *M. avium*, serovar 2, 3 showed weak growth at 45 °C in only 14 % of the strains, compared with 91 % of avirulent strains having the characteristics of *M. intracellulare* serovar (Table 4).

Table 3

Thermoresistance of M. avium — M. intracellulare compared with the virulence for pullets

Bioassay on pullets	No. of strains	Growth of strains after exposure to 60 °C for							
		30 minutes		1 hour		2 hours		4 hours	
		pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.
Negative	82 100 %	82 100 %	0	82 100 %	0	75 91,4 %	7 8,6 %	6	76 92,7 %
Positive	59 100 %	59 100 %		59 100 %		2 3,4 %	57 96,6 %		59 100 %

Table 4

Growth of M. avium — M. intracellulare strains in serum medium at 22 °C and 45 °C

Mycobacterium species	No. of strains	Serum medium — incubation							
		1 month				2 months			
		22 °C		45 °C		22 °C		45 °C	
		+	−	+	−	+	−	+	−
M. avium	7 100 %	0	7 100 %	1 14 %	6 86 %	4 57 %	3 43 %	1 14 %	6 86 %
M. intracellulare	22 100 %	3 14 %	19 86 %	19 86 %	3 14 %	17 77 %	5 23 %	20 91 %	1 4 %

Discussion

In the thermoresistance tests of mycobacteria with the capillary tube technique the reference strains of *M. intracellulare* proved less thermoresistant than *M. intracellulare* field strains isolated from pigs. The differences in the thermoresistance of mycobacteria of the *M. avium — M. intracellulare* complex could be utilized for assessment of their virulence which would limit, to a considerable extent, ex-

pensive bioassays used for this purpose. The results reported in the present study are also of value to food hygiene. We recommend that thermoresistance tests of mycobacteria should be used in comprehensive evaluation of the characteristics of some pathogenic mycobacterial species.

Termorezistence mykobaktérií

Byla sledována termorezistence některých druhů patogenních mykobaktérií ve vodném prostředí a v tekuté sérové půdě. Metodou skleněných kapilár byla prověřena termorezistence 105 kmenů 7 druhů mykobaktérií *(M. bovis, M. avium, M. intracellulare, M. gordonae, M. kansasii, M. smegmatis, M. phlei.)* Všechny kmeny byly vystaveny teplotám 60, 65, 70 a 75 °C a expozici 10, 20, 40 sekund, 2, 4, 8, 16, 30, 60 minut a 2, 4, 6, 8 hodin. Nejmenší termorezistence byla zjištěna u kmenů *Mycobacterium bovis,* které byly devitalizovány při 60 °C již při šestnáctiminutové expozici a při teplotě 70 a 75 °C do 10 sekund. Největší termorezistenci vykazovaly kmeny *Mycobacterium phlei,* které byly devitalizovány při 75 °C a dvacetisekundové expozici.

Pomocí jednoduché a snadno reprodukovatelné zkumavkové metody a tekutou sérovou půdou pro kultivaci mykobaktérií byly zjištěny průkazné rozdíly v termorezistenci při 60 °C a dvouhodinové expozici kmenů mykobaktérií komplexu *M. avium — intracellulare* v závislosti na jejich virulenci při biologických pokusech na drůbeži. Kmeny virulentní (*M. avium,* sérovar 2, 3) vykazovaly nižší rezistenci ve srovnání s kmeny avirulentními. Uvedené výsledky byly v korelaci s hodnocením růstu kmenů komplexu *M. avium — intracellulare* při rozdílných teplotách.

Z výsledků studia termorezistence vyplývá, že uvedená metoda umožňuje rozšířit poznatky o biologických vlastnostech jednotlivých druhů mykobaktérií.

Теплоустойчивость микобактерий

Проводились исследования возможности определения видовой принадлежности некоторых патогенных микробактерий с помощью их теплоустойчивости в водяной среде. Методом стеклянных капилляр проверяли теплоустойчивость 105 штаммов 7 видов микобактерий *(M. bovis, M. avium, M. intracellulare, M. gordonae, M. kansasii, M. smegmatis, M. phlei).* Все штаммы подвергались температуре 60, 65, 70 и 75 °С с выдержкой 10, 20, 40 секунд, 2, 4, 8, 16, 30, 60 минут и 2, 4, 6, 8 часов. Самая малая теплоустойчивость была выявлена у штаммов *Mycobacterium bovis,* умерщвленных при 60 °С уже при выдержке 16 минут, при 65 °С с выдержкой двух минут и при температуре 70 и 75 °С до 10 секунд. Самой большой теплоустойчивостью отличались штаммы *Mycobacterium phlei,* умерщвленные при 75 °С с выдержкой 20 секунд. Существенная разница теплоустойчивости была выявлена у штаммов *Mycobacterium avium* и *Mycobacterium intracellulare.* При температуре 60 °С с выдержкой 60 минут были умерщвлены все штаммы *M. intracellulare,* между тем как все культуры *M. avium* выживали при упомянутой температуре и выдержке. Аналогичная существенная разница была уста-

новлена также в ходе оценки теплоустойчивости штаммов *Mycobacterium bovis* и *Mycobacterium kansasii.* Из результатов изучения теплоустойчивости вытекает, что приведенный метод способствует расширению знаний относительно свойств отдельных видов микобактерий.

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