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Research Note

Real-Time Quantitative PCR Detection of *Mycobacterium avium* Subspecies in Meat Products

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ABSTRACT

The aim of this work was to examine various purchased meat products and to find out if any traces of *Mycobacterium avium* subsp. *avium, M. avium* subsp. *hominissuis,* and *M. avium* subsp. *paratuberculosis* could be detected in these samples. Analysis of the meat products (raw, cooked, and fermented) was performed using a real-time quantitative PCR (qPCR) method for the detection of specific insertion sequences: duplex qPCR for the detection of IS*900* specific for *M. avium* subsp. *paratuberculosis,* and triplex qPCR for the detection of IS*901* specific for *Mycobacterium avium* subsp. *avium* and IS*1245* specific for *M. avium* subsp. *hominissuis*. Of the 77 analyzed meat samples, 17 (22%) were found to contain *M. avium* subsp. *paratuberculosis* DNA, 4 (5%) samples contained *Mycobacterium avium* subsp. *avium* DNA, and in 12 (16%) samples *M. avium* subsp. *hominissuis* DNA was detected. The concentration of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* DNA in some meat products exceeded 10^4 genomes per g. Culture examination of these mycobacterial subspecies was negative. By analyzing a range of meat products, we have provided evidence to support the hypothesis that *M. avium* is present in everyday commodities sold to the general public.

The zoonotic significance of *Mycobacterium avium* subspecies is still being discussed by many research groups today *(19)*. *M. avium* is divided into four subspecies, specifically, *M. avium* subsp. *avium, M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis,* and *M. avium* subsp. *silvaticum*. They can cause diseases such as tuberculosis-like illness, disseminated infections, osteomyelitis, and lymphadenitis in immunocompromised patients and mammals *(8)*. *M. avium* subsp. *paratuberculosis* causes paratuberculosis in ruminants (Johne's disease), while its association with Crohn's disease as yet remains unproven *(4)*. *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* can be spread into the environment and into animals by various routes and can eventually come into contact with people. The major source of *M. avium* subsp. *hominissuis* is considered to be soil, from which the organisms are washed away into rivers *(6)*; nevertheless, *M. avium* has been detected in foods such as vegetables, milk, and dairy products *(2, 14)*.

Very few surveys have been performed to determine the presence of *M. avium* subsp. *avium, hominissuis,* and *paratuberculosis* in meat products. Nevertheless, the results confirmed meat as a possible source for the transmission of potentially pathogenic mycobacteria to humans *(16)*. Alonso-Hearn et al. *(1)* detected and cultured *M. avium* subsp. *paratuberculosis* from the muscle tissue of infected cattle and suggested a potential risk of exposure to humans via contaminated meat. The heat resistance of *M. avium* in sausages has also been confirmed *(15)*. However, analysis of ground beef did not reveal the presence of *M. avium* subsp. *paratuberculosis (10)*. In addition, it was stated that any *M. avium* subsp. *paratuberculosis* detected in muscle, lymphatic, and organ tissues from cows with advanced paratuberculosis is likely to be inactivated when the meat is cooked to a ''well done'' consistency *(13)*. The heat treatment of mycobacterial cells in meat has also been studied *(11, 12)*, with the results showing a significant decrease in risk to the consumer after treatment.

The aim of this work was to examine a range of meat products purchased in three supermarkets and in one butcher shop by quantitative real-time PCR (qPCR) and culture, to determine the presence of *M. avium* subspecies *paratuberculosis*, *avium,* and *hominissuis* in these products.

MATERIALS AND METHODS

Origin and storage of samples. Overall, 77 meat products were purchased from three supermarkets and one butcher shop in a city in the Czech Republic and stored at −70°C. They were then divided into three groups: 54 (70%) raw products, five (7%) cooked products, and 18 (23%) fermented meat products. The meat products were then further differentiated into subgroups according to their origin (bones, meat and meat on bones, offal, minced meat, salami, ham, and sausage; Tables 1 and 2).

Isolation of DNA. DNA from the meat samples was isolated as previously described by Slana et al. *(17)*. Fifty milligrams of the samples was processed according to a slightly modified protocol based on a commercially available kit (DNeasy Blood & Tissue kit,

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TABLE 1. *Detection of* Mycobacterium avium *subsp.* paratuberculosis, *subsp.* avium, *and subsp.* hominissuis *in raw meat products examined by quantitative real-time PCR and culture*[a]

Process	Origin	Name	No. of samples examined/no. positive	qPCR *M. avium* subsp. *paratuberculosis* IS*900* (copies/g)	Triplex qPCR *M. avium* subsp. *avium* IS*901* (copies/g)	Triplex qPCR *M. avium* subsp. *hominissuis* IS*1245* (copies/g)	Cultivation[b]
Raw	Beef	Bones	1/0	−	−	−	−
		Meat	7/1	2.53×10^0	−	2.88×10^2	+[c]
		Meat on bone	2/1	2.92×10^3	−	−	−
		Offal	1/0	−	−	−	−
		Minced meat	1/0	−	−	−	−
	Pork	Bones	1/0	−	−	−	−
		Meat	2/0	−	−	−	+[c]
		Meat on bone	7/2	5.97×10^2	−	2.41×10^3	−
		Offal	6/0	−	−	−	−
		Salami	4/2	−	−	1.74×10^2	−
				−	−	6.14×10^2	−
		Ham	1/1	5.06×10^2	−	−	+[c]
		Sausage	3/2	4.84×10^3	−	−	−
				3.58×10^3	−	1.12×10^4	−
		Minced meat	2/0	−	−	−	+[d]
	Chicken	Meat on bone	4/2	2.72×10^2	−	−	−
				6.34×10^2	−	−	−
		Meat	4/3	7.41×10^2	−	−	−
				9.33×10^2	−	−	−
				7.72×10^2	−	−	−
		Offal	2/0	−	−	−	−
	Duck	Offal	2/0	−	−	−	+[c]
	Lamb	Meat	2/1	−	−	5.52×10^3	−
	Mix	Minced meat	2/1	3.74×10^2	−	−	+[c]
Total			54/16				

[a] −, negative results.
[b] Published previously *(16)*.
[c] Identified by sequencing as *M. chelonae (16)*.
[d] *Mycobacterium* species.

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TABLE 2. *Detection of* M. avium *subsp.* paratuberculosis, *subsp.* avium, *and subsp.* hominissuis *in cooked and fermented meat products examined by quantitative real-time PCR (qPCR) and culture*[a]

Process	Origin	Name	No. of samples examined/no. positive	qPCR *M. avium* subsp. *paratuberculosis* IS*900* (copies/g)	Triplex qPCR *M. avium* subsp. *avium* IS*901* (copies/g)	Triplex qPCR *M. avium* subsp. *hominissuis* IS*1245* (copies/g)	Cultivation[b]
Cooked	Beef	Offal	1/1	2.63×10^2	–	4.91×10^2	+[c]
	Pork	Meat loaf	1/1	5.35×10^3	–	2.02×10^3	–
		Meat on bone	1/0	–	–	–	–
		Salami	2/1	–	2.96×10^2[d]	–	+[c]
Subtotal			5/3				
Fermented	Pork and beef	Salami	16/7	1.08×10^4	2.36×10^2	–	–
				2.43×10^4	8.08×10^1	–	–
				–	–	3.53×10^3	–
				1.39×10^4	–	–	–
				–	–	4.35×10^2	–
				–	–	2.25×10^4	–
				–	4.18×10^0[e]	–	–
		Sausage	2/1	–	–	3.42×10^2	–
Subtotal			18/8				
Total			23/11				

[a] –, negative results.
[b] Published previously *(16)*.
[c] Identified by sequencing as *M. chelonae (16)*.
[d] In this sample, 4.88×10^4 copies of IS*1245* per gram was also detected.
[e] In this sample, 4.96×10^5 copies of IS*1245* per gram was also detected.

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Qiagen, Hilden, Germany). The isolated DNA was used as a template in duplex and triplex qPCR.

qPCR. For the detection of a specific insertion sequence (IS*900* explicit for *M. avium* subsp. *paratuberculosis*), duplex qPCR with an internal amplification control was used for all samples as described by Slana et al. *(18)*. For the detection of specific insertion sequences IS*901* (specific for *M. avium* subsp. *avium*) and IS*1245* (specific for *M. avium* subsp. *hominissuis* and subsp. *avium*), triplex qPCR with an internal amplification control was used for all samples as described by Slana et al. *(17)*.

Culture. One gram of the sample was homogenized and decontaminated in 1 M HCl and then neutralized with 2 M NaOH. Eighty microliters of the suspension was inoculated onto two solid media (Herrold's egg yolk medium and medium according to Stonebrink) and one liquid medium (according to Sula), as described by Fischer et al. *(7)*.

RESULTS

Of the 77 meat samples analyzed, 17 (22%) were found to contain *M. avium* subsp. *paratuberculosis* DNA, 4 (5%) samples contained *M. avium* subsp. *avium* DNA, and in 12 (16%) samples *M. avium* subsp. *hominissuis* DNA was detected (Tables 1 and 2).

In the raw products group, 12 (22%) samples contained *M. avium* subsp. *paratuberculosis* DNA, none of the raw products contained *M. avium* subsp. *avium* DNA, and in 6 (11%) raw products *M. avium* subsp. *hominissuis* DNA was detected (Table 1). Three cooked meat products were positive for *M. avium*; of these, two (40%) contained *M. avium* subsp. *paratuberculosis* and subsp. *hominissuis* DNA, and in one (20%) sample, *M. avium* subsp. *avium* DNA was found (Table 2). Fermented meats contained *M. avium* subsp. *paratuberculosis* DNA in three (17%) products, three (17%) products contained *M. avium* subsp. *avium* DNA, and four (22%) contained *M. avium* subsp. *hominissuis* DNA (Table 2).

Culture results of raw and processed meat products showed no positivity for any *M. avium* subspecies. There were eight mycobacterial isolates, which were further identified by sequencing according to Harmsen et al. *(9)* as *Mycobacterium chelonae,* except for one that was identified as *Mycobacterium* species (Tables 1 and 2). These results have been formerly published in detail *(16)*.

DISCUSSION

In previous studies, the qPCR method has been proven to be a rapid and sensitive method for the detection of *M. avium (17, 18)*. In our survey we have displayed the presence of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium,* and/or *M. avium* subsp. *hominissuis* DNA in 40% of the purchased meat products analyzed. The same meat samples were used in a study by Shitaye et al. *(16)*; however, their culture method did not prove the presence of any *M. avium* subspecies. A possible explanation could be the presence of dead or viable but noncultivable cells *(3)*.

In our study, there were no major differences in the amounts of mycobacteria detected in raw and in processed meats. Nevertheless, this suggestion needs further research. The sterilization processes that meat products undergo during production, e.g., heat treatment, are not sufficient for the destruction of DNA; therefore, we were able to detect mycobacterial DNA by qPCR in the amounts stated in Tables 1 and 2. So far, published results only report counts of viable cells *(11, 12)*.

Distinct differences in the amounts of mycobacterial DNA detected in groups according to meat origin were observed in raw meats, meat on bones, fermented products, salami, and sausages specifically. Savov *(15)* confirmed the presence of *M. avium* subsp. *avium* in dried and fumigated sausages. These results also support a previous hypothesis, that heat inactivation of mycobacteria, especially in fermented products, may be insufficient to ensure safety. Jaravata et al. *(10)* surveyed 200 ground beef samples by multiplex qPCR and found the tested samples to be negative for the presence of *M. avium* subsp. *paratuberculosis*. In our study, we examined six minced meat samples (five raw products and one cooked product, meat loaf) and found two products to be positive for *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis* DNA (Tables 1 and 2).

The positive results obtained from the tested meat samples may be caused by preslaughter conditions, the cleanliness of equipment, staff handling, and general standards of technological processing, which are all significant agents affecting the final microbiological quality of the products *(5)*. This could be a possible explanation for finding *M. avium* subsp. *paratuberculosis* and subsp. *hominissuis* DNA in five of the samples, as well as double contamination of *M. avium* subsp. *paratuberculosis* and subsp. *avium* DNA in two samples.

By analyzing various raw and processed meat products, we have provided evidence of the presence of DNA of multiple *M. avium* subspecies in commercially and publicly available consumable goods. This may thus represent a potential pathway for transmission to humans.

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