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A novel tube-in-tube filter system for refinement of DNA preparation for detection of Mycobacterium *avium* subspecies *paratuberculosis* in bovine feces using Real-Time PCR

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Abstract

A novel tube-in-tube filter system, the AutoLys M 1.0 tube with a 1 µm pore size non-DNA binding and RNase and DNase free filter designed and produced by HAMILTON Bonaduz AG could be successfully integrated in the procedure of DNA extraction from fecal samples for testing on Mycobacterium avium subsp. paratuberculosis (MAP) in Real-Time PCR. The results obtained indicate an increase in sensitivity of MAP detection in feces. The implementation of this filter tube system means a decisive step towards standardization and high-throughput detection of MAP in fecal samples as an alternative to time and labor intensive culture.

Introduction

In the diagnosis of Johne's disease, isolation of the pathogen agent *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by cultivation has been increasingly superseded by direct detection of MAP using Real-Time PCR (Leite et al., 2013; Okwumabua et al., 2010; Park et al., 2014; Selim and Gaede, 2012). The reasons for this development include the long lasting cultivation of MAP and also improved methods of DNA preparation and PCR protocols for sensitive and specific pathogen detection (EFSA, 2004; Logar et al., 2012).

However, sensitive detection of MAP in feces using PCR is still cumbersome and challenging due to inhibitors, low pathogen numbers, clustering of the MAP bacteria in feces and high stability of MAP cells hampering DNA extraction (Bull et al., 2003; EFSA, 2004; Plain et al., 2014; Wilson, 1997). A recently published study (Sting et al., 2014) describes the reduction of inhibitors and concentration of the MAP bacteria by the integration of a filtration step in the extraction procedure of MAP DNA from fecal suspensions leading to increased detection rates.

The aim of the present study is to facilitate, standardize and refine this filtration procedure by using a novel filter tube system as a decisive step in the extraction of DNA from fecal samples for testing in MAP PCR.

Material and methods

A total of 38 bovine fecal samples were included in this study. For comparative studies, the procedure described previously (Sting et al., 2014) and based on the method specified by Selim and Geaede (2012) served as standard. Suspension of 1 g feces in 5 ml citrate buffer (Diagnostica Stago, Asnières-sur-Seine, France) was followed by centrifugation at 1,000xg for 1 min. The inner tube of the tube-in-tube system Hamilton AutoLys M 1.0 tube with a 1 µm pore size and a non-DNA binding membrane produced free of detectable RNase and DNase activity (HAMILTON Bonaduz AG, Switzerland) (Figure 1B) was filled with 200 µl of the recovered supernatant which had previously been diluted with 300 µl citrate buffer (Diagnostica Stago). The Hamilton AutoLys M 1.0 tubes were centrifuged at 6,000xg for 2 min in a fixed angle rotor (Universal 32R, Hettich, Tuttlingen, Germany) or at 5,000xg for 10 min in a swing-out rotor (Rotina 380R, Hettich, Tuttlingen Germany) with the inner tube lifted in the lift and lock position (Figure 1D). After centrifugation, the supernatant and the flow-through were discarded. In a further step, 150 mg beads (e.g. 0.1 mm zirconia/silica beads, Roth, Karlsruhe, Germany) and 500 µl citrate buffer (Diagnostica Stago) were added directly into the inner tube of the Hamilton AutoLys M 1.0 tube system in the parking position (Figure 1A).

Mechanical disruption of MAP was carried out in the Hamilton AutoLys M 1.0 tube

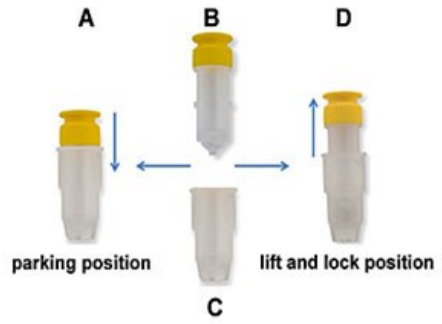

Fig. 1: The Hamilton AutoLys M 1.0 tube (A) consists of an inner tube (B) with 1.0 µm non-DNA binding membrane and a collection (outer) tube (C) with a 2D barcode on the bottom. The tube can be used in parking positive (A) (e.g. for mechanical disruption, heat treatment) or in lift and lock position (D) (e.g. for centrifugation).

(parking position) in the TissueLyser (Qiagen, Hilden, Germany; 3 x 3 min, 30/sec) using a 24 well rack (HAMILTON Bonaduz AG, Switzerland) (Figure 2) or in the Precellys 24® (bertin technologies, Saint Quentin en Yvelines Cedex, France, with a prototype adapter for six AutoLys M 1.0 tubes; 3 x 60 sec, frequency 6,500 rpm).

Fig. 2: AutoLys 24 well rack in SBS format for centrifugation of the Hamilton AutoLys M 1.0 tubes with the inner tube in lift and lock position (left) or for mechanical disruption of the MAP bacteria in the Hamilton AutoLys M 1.0 tubes in parking position (right).

In a further step, the suspensions containing the disrupted MAP bacteria were heated directly in the Hamilton AutoLys M 1.0 tube with the inner tube in parking position (Figure 1A). Heating took place in a heater

(ThermoStat plus, Eppendorf, Hamburg, Germany) at 95 °C for 30 min in an exchangeable Hamilton thermoblock adapter (HAMILTON Bonaduz AG, Switzerland) (Figure 3).

Fig. 3: Exchangeable Hamilton thermoblock adapter for heating of the MAP-suspension in the Hamilton AutoLys M 1.0 tubes (the Hamilton heating block has to be mounted on an Eppendorf Thermomixer comfort or ThermoStat plus heater).

After centrifugation of the Hamilton AutoLys M 1.0 tubes with the inner tube in parking position at 5,000xg for 5 min (Rotina 380R, Hettich), 200 µl of the supernatant was processed as described by Selim and Gaede (2012) using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The procedure started with lysozyme digestion according to the manufacturer's instructions specified in the chapter "Isolation of nucleic acids from bacteria or yeasts". This kit was carried out in a manual or semi-automated manner using a vacuum system on the STARlet pipet robot (HAMILTON, Bonaduz, Switzerland) for DNA extraction. The automated procedure started with the addition of isopropanol. Elution of DNA was carried out using a volume of 100 µl.

In further comparative studies, the sample volume used for the Hamilton AutoLys M 1.0 tube was increased fourfold (800 µl of the fecal supernatants + 1,200 µl citrate buffer), or an additional washing step (2,000 µl citrate buffer) and a centrifugation step (6,000xg, 2 min) were included before the mechanical disintegration of the MAP bacteria.

In the main study, the procedure described by Sting et al. (2014) was applied as the standard method and compared with the procedure including the Hamilton AutoLys M 1.0 tube as described above with a centrifugation step at 6,000xg for 2 min in a fixed angle rotor (Universal 32R, Hettich). In order to process the samples quickly, further studies were performed using the Qiagen TissueLyser for mechanical disruption.

Detection of MAP DNA was performed by Real-Time PCR as specified by Kim et al. (2002), targeting the IS900 gene of MAP. PCR runs were carried out on the PCR cycler CFX96 Real-Time (BioRad, Munich, Germany) according to the conditions specified by Sting et al. (2014). For detection of PCR inhibitors, an internal control system based on the IC2 plasmid was included (Hoffmann et al., 2006). The samples were tested in triplicate and CT mean values calculated.

Tab. 1: Comparative studies on the centrifugation of the sample loaded Hamilton AutoLys M 1.0 tube.

Sample No.	Fixed angle rotor (C_T values)	Swing-out rotor (C_T values)	Differences in C_T values
1	29.99	29.23	0.76
2	30.05	29.56	0.49
3	28.34	27.62	0.72
4	31.65	30.69	0.96
5	28.21	27.28	0.93

Results

In preliminary studies, the effect of the devices used for centrifugation of the filters and disruption of MAP cells on the success of DNA extraction was examined. Furthermore, the influence of an increased sample load and an additional washing and centrifugation step on MAP detection by Real-Time PCR were examined. The method described by Sting et al. (2014) served as standard.

The use of a fixed angle rotor carrying six samples (6,000xg) could be replaced by a slower rotating speed swing-out rotor (5,000xg instead of 6,000xg) which can hold 24 samples. The slower rotation speed of the rotor could be compensated by a prolonged centrifugation time (Table 1).

Comparative studies on the method described by Sting et al. (2014) (standard method), the TissueLyser (Qiagen), and in a further step the Precellys 24® (bertin technologies) for mechanical disruption of the MAP cells, showed comparative results for all procedures, irrespective of the filter system or homogenizer used (Table 2a). However, in comparison to the method described by Sting et al. (2014), in four of five samples the PCR signals were stronger with the Hamilton AutoLys M 1.0 tube in the TissueLyser (Qiagen), leading to differences in CT values of 1.3 to 1.9 (Table 2a). Comparing the use of Hamilton AutoLys M 1.0 tubes in the TissueLyser (Qiagen) and in the Precellys 24® homogenizer (bertin technologies), the last-mentioned provided slightly to clearly better results for three samples (difference of CT values of 1.08 to 2.27) (Table 2b). In the main study, the TissueLyser (Qiagen) was preferred due to processing of more samples in one run. The Precellys 24® homogenizer (bertin technologies) was produced as a prototype providing only six sample positions. However, a technically modified type would be able to allow the processing of more samples per run.

Increasing the sample volume by a factor of four led to stronger PCR signals in three cases (difference of CT values of 2.5 to 4.2), but in two cases to a significant decrease in PCR signals (difference of CT value of 7.0 and in one sample no signal detectable due to PCR inhibition).

Tab. 2a: Comparative studies on mechanical disruption of MAP cells using different devices and filter systems. Standard method = method described by Sting et al. (2014)

Sample No.	Method A Standard method (C_T values)	Method B Standard method using the TissueLyser (Qiagen) (C_T values)	Method C Hamilton AutoLys M 1.0 tube using the TissueLyser (Qiagen) (C_T values)	Differences in C_T values Method A and B	Differences in C_T values Method A and C
6	21.58	22.72	19.96	1.14	1.62
7	27.01	28.14	25.10	1.13	1.91
8	30.24	29.39	28.94	0 .85	1.30
9	29.82	30.63	29.70	0.81	0.12
10	25.39	26.43	23.93	1.04	1.46

Tab. 2b: Comparative studies on mechanical disruption of MAP cells using the TissueLyser (Qiagen) and the Preyellys 24® (bertin technologies).

Sample No.	Method C Hamilton AutoLys M 1.0 tube using the TissueLyser (Qiagen) (C_T values)	Method D H amilton AutoLys M 1.0 tube using the Precellys 24® (bertin technologies) (C_T values)	Differences in C_T values Method C and D
32	32,91	32,23	0,68
37	31,47	29,20	2,27
38	31,20	29,51	1,69
40	37,14	37,59	0,45
41	33,17	32,09	1,08

An additional washing and centrifugation step with the aim of reducing PCR inhibitors was not successful, and led to a significant reduction in PCR signals in all ten samples tested (difference of CT values of 2.0 to 6.1) (data not shown).

The main study included 30 samples and aimed to compare the micro-spin filter and the Hamilton AutoLys M 1.0 tube, both of which were included in the procedure described by Sting et al. (2014). The results obtained were comparable, however the Hamilton AutoLys M 1.0 tubes achieved stronger positive signals in PCR in all except for one sample, leading to differences in CT values as high as 5.8 (Table 3).

Discussion

Refined protocols for DNA extraction from feces combined with Real-Time PCR have decisively improved the diagnosis of Johne's disease. Based on a sophisticated protocol described by Selim and Gaede (2012), integration of an additional filtration step in the procedure for processing fecal samples for PCR is suitable to further improve the sensitivity of MAP detection by the concentration of MAP cells and removal of PCR inhibitors (Sting et al., 2014). However, in diagnostic procedures standardization is crucial to provide valid results (Plain et al., 2014). Optimization and standardization are of particular concern in view of achieving a robust and reliable test procedure. Implementation of a method in routine diagnostics also has to consider aspects of turnaround time and throughput of test samples required (OIE, 2012; Plain et al., 2014). To meet these demands, the novel Hamilton AutoLys M 1.0 tube was used instead of a micro-spin filter. The use of the Hamilton AutoLys M 1.0 tube allows a concentration of MAP, removing of PCR inhibitors and mechanical and thermal disintegration of MAP cell walls in combination with automation of the DNA extraction procedure. In addition, refinement by simplification and standardization of the filtration step within the procedure of processing of fecal samples means a further step towards high-throughput diagnosis of Johne's disease. Furthermore, the Hamilton AutoLys M 1.0 tube can be used for concentration of MAP cells cultivated in supernatants of fecal suspensions, milk samples or fluid media, which means a promising approach towards an increase in sensitivity of MAP testing. Altogether, this might result in a routine procedure for detection of MAP in feces by Real-Time PCR being competitive against methods of cultivation (Chui et al., 2004; Leite et al., 2013, Okwumabua et al., 2010; Plain et al., 2014; Selim and Gaede, 2012; Sting et al., 2014).

The use of the Hamilton AutoLys M 1.0 tube for testing of liquid samples such as milk or liquid MAP cultures should be investigated in more detail in further studies.

Conflict of interest statement

HAMILTON Bonaduz AG did not play any role in conducting the study design or analysis and interpretation of data. None of the authors of the CVUA Stuttgart have any financial or personal relationships that could inappropriately influence or bias the content of this article.

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Tab. 3: Results of the main study comparing procedures for extraction of DNA from bovine fecal samples for MAP Real-Time PCR. Centrifugation at 6,000xg for 2 min in a fixed angle rotor. Standard method = method described by Sting et al. (2014)

Sample No.	Standard method (C_T values)	Hamiltin AutoLys M 1.0 tube (C_T values)	Difference in C_T values (C_T values)
11	30.61	28.46	2.15
12	28.84	28.61	0.23
13	36.64	32.87	3.77
14	33.01	31.16	1.85
15	27.88	27.16	0.72
16 (= 9)	30.63	29.70	0.93
17 (= 10)	26.43	23.93	2.50
18	35.42	29.61	5.81
19	35.09	32.09	3.00
20	25.13	24.43	0.70
21	34.78	32.17	2.61
22	34.54	32.09	2.45
23	35.98	34.81	1.17
24	Inhibition	39.56	Inhibition
25	32.54	30.31	2.23
26	35.74	32.87	2.87
27	36.10	Inhibition	Inhibition
28	34.87	34.86	0.01
29	29.12	27.54	1.58
30	39.37	41.64	2.27
31	37.78	37.60	0.18
32	33.78	32.91	0.87
33	39.50	37.82	1.68
34	Inhibition	37.42	Inhibition
35	Inhibition	40.41	Inhibition
36	39.19	38.12	1.07
37	35.16	31.47	3.69
38	32.44	31.20	1.24
39	40.68	37.14	3.54
40	33.16	33.17	0.01