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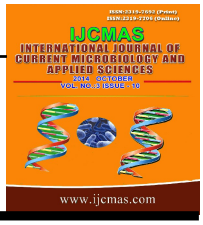

Original Research Article

Evaluation of conventional and real-time PCR assays for Molecular diagnosis of Johne's disease in dairy cattle

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Keywords

Conventional PCR; Real-time PCR, IS900; F57; Johne's disease; Map

A B S T R A C T

Mycobacterium avium subsp. paratuberculosis (Map) is the etiologic agent of Johne's disease, a disease with considerable economic impact on dairy cattle herds. The infected animals shed viable *Map* in their milk and faecas. The purpose of this study was to evaluate both the IS900 and F57 segments as a target for detection of *Map* DNA in clinical samples using conventional PCR and IS900 real-time PCR assays for diagnosis of Johne's disease in dairy cattle. Detection limits of conventional PCR assay using IS900 and F57 primer sets were $1.1x10^{-6}$ µg/µl and $1.1x10^{-12}$ µg/µl, respectively. However, by using IS900 real-time PCR, the detection limit was found to be $1.1x10^{-8}$ µg/µl of pure DNA which corresponds to $2.2x10^{-6}$ genomic copy. Out of 150 faecal samples, 31.33%, 29.33% and 42.66% were found positive by conventional IS900 PCR, conventional F57 PCR and IS900 Real-time PCR, respectively. Considering milk samples, *Map* DNA was successfully detected in 4.67%, 3.33% and 5.33% samples by IS900 conventional PCR, F57 conventional PCR and IS900 real-time PCR assays, respectively. In conclusion, real-time PCR was significantly more sensitive in direct detection of *Map* DNA in cattle faecal samples than conventional PCR assays. There was no significant difference between the IS900 and F57 primer sets when been used in direct detection of *Map* in clinical samples. For diagnosis of *Map* by different PCR assays, faecal samples were confirmed to be the optimum samples versus milk samples.

Introduction

Paratuberculosis (PTB, Johne's disease) is nowadays viewed as one of the most serious chronic bacterial diseases of ruminants such as cattle, sheep and goats (Stabel *et al*., 2004; Fernández-Silva *et al*., 2014). The disease is also found in wild animals (Machackova *et al*., 2004; Fischer *et al*., 2005). World-wide, the prevalence of the disease can range from 3-4% of the herds in regions with low incidence such as England (Cetinkaya *et al*., 1996), to 70.4% of cattle dairy operations in regions of high incidence such as the United States (Lombard *et al*., 2013). In the most of the African countries, at least one incident of the disease or the disease was suspected in cattle, sheep or

goats (OIE, 2009). Despite the assumed presence of this disease among veterinary practitioners and farmers in Egypt for long time, only a dearth of information is available about the impact distribution, prevalence, epidemiology and economic losses of Johne's disease on dairy cattle. The etiologic agent of PTB, *Mycobacterium avium subsp. paratuberculosis* (*Map*) is a slowly growing, mycobactin-dependent acid-fast bacterium (Sweeney, 1996). *Map* is resistant to environmental conditions (Grant, 2005). On the other hand, the role of *Map* in public health as the causative agent of Crohn's disease (CD) in human has proven in some reports (Ghadiali *et al*., 2004; Sechi *et al*., 2005) based on similar clinical signs and pathology to paratuberculosis and denied by others (Freeman and Noble, 2005; Romero *et al*., 2005).

The primary source of *Map* transmission to any calf is faecal contamination of milk and feed (Sweeney, 1996). The calf may also get infected from symptomatic or asymptomatic mother in utero (Seitz *et al*., 1989). There is also potential for *Map* transfer between calves especially if they are group housed (Mortier, *et al*. 2014). After long incubation period, the infected cows may have clinical signs such as persistent diarrhoea and the irreversible progressive weight loss (Hasonova and Pavlik, 2006). However, asymptomatic animals may shed *Map* with colostrum and milk (Sweeney *et al*., 1992; Streeter *et al*., 1995).

Many diagnostic tests were used for diagnosis of PTB in cattle. The bacterioscopic method using Ziehl-Neelsen stain usually used for PTB identification. This method has the advantage of being simple, fast and inexpensive, but has the disadvantage of having very low sensitivity and specificity in faecas, colostrum and milk samples. The detection of antibodies for *Map* by enzyme-linked immunosorbent assay (ELISA) is one of the important serological diagnostic assay (Shahmoradi *et al*., 2008). Although ELISA is fast, inexpensive and easy to implement, it has the disadvantage of being variable in individual responses depending upon the stage of disease (Manning and Collins, 2001). Inaccurate identification and poor sensitivity of bacterial culture (15-25 %) is due to lack of shedding in early phases, intermittent shedding, limitations of culture protocols and the disadvantages of solid culture media (Metzger, *et al*., 2006).

Because of the disadvantages of the afore-mentioned classical tests for diagnosing the disease, it is necessary to consider other rapid, sensitive and specific diagnostic tests. Recently, the characterization of the IS*900* and F57 insertion sequences (Collins *et al*., 1989; Paustian *et al*., 2010) have enabled the specific identification of minimum amounts of bacterial DNA by different polymerase chain reaction (PCR) techniques (Rodriguez-Lazaro *et al*., 2005; Stephan *et al*., 2007). Therefore, the main aim of this study was to evaluate fast and sensitive conventional and real-time PCR protocols for diagnosis of Johne's disease in dairy cattle. .

Material and Methods

Samples

One hundred and fifty faecal and milk samples were collected from less than 2 years old imported holstein and Friesians cows from USA, Holland and Germany with and without clinical symptoms of paratuberculosis (chronically or intermittent diarrhoea and weight loss). The samples were kept at 4^{o}C until processed for DNA extraction, IS900 and F57 PCR assays within 24 hours from their collection.

Pure DNA of *Map* K10 strain was kindly provided by Prof. Dr.Talaat, at University of Wisconsin-Madison Madison, WI, USA. It was used in the sensitivity experiment and as a positive control

DNA extraction

Mycobacterium avium subsp. paratuberculosis DNA was extracted from the faecal samples according to methods described previously by Stabel *et al*. (2004) and from milk samples according to Paolicchi *et al*. (2003). Five microlitres from each prepared sample was used for the PCR assay.

IS900 and F57 conventional PCR assay

DNA was amplified by PCR using specific IS900 primers (IS900/150C 5`-CCG CTA ATT GAG AGA TGC GAT TGG-3`, forward primer, and IS900 921 5` AAT CAA CTC CAG CAG CGC GGC CTC G-3`, backward primer) as described previously (Stabel and Bannantine, 2005) and for F57 PCR assay using F57 specific primers (F57 forward 5'-GCC CAT TTC ATC GAT ACC C-3' and F57 reverse 5'-GTA CCG AAT GTT GTT GTC AC-3') according to Slana *et al*. (2008). Genomic DNA of *Map* K10 strain was included during each round of PCR performed as a positive control. Amplification products were subjected to electrophoresis in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min and visualized under ultraviolet light. Presence of 229 bp band for IS900 and 147bp band for F57 primer indicated the presence of *Map* DNA in the sample.

IS900 real-time PCR assay

DNA was amplified by real-time PCR in a total volume of 20 µl using the IS900 primer pair. The amplification reaction consists of 1X ABsolute™ QPCR SYBR Green Mix, Thermoscientific, ABgene, UK, 10 pmol of each primer and 5 µl of DNA as template. Negative control consisted of reaction mixture alone and positive control contained 1 µl of genomic DNA of *Map* K10. Reaction condition for IS900 according to Stabel *et al*. (2004) was used. Melt curve analysis was performed by heating the PCR products from 60 ºC to 95 ºC for 2 min.

Relative sensitivities of conventional and real-time PCR assays

The relative sensitivities of different PCR assays were determined by amplification of 10-fold serial dilutions of pure DNA of K10 strain of *Map* starting from 1.1µg/µl to 10^{-15}µg/µl which corresponded to 220 genomic copies of DNA to 2^{-12} genomic copy (Stabel *et al*., 2004; Stabel and Bannantine, 2005).

Statistical analysis

The results of all PCR assays were analyzed by using Chi-Square analysis (X^2**)** to detect the most sensitive PCR assay in detection of *Map* in clinical samples (faecal and milk samples).

Results and Discussion

Map is one of the most important agents that cause several economic losses (Nielsen and Toft, 2009). Therefore, early diagnosis of Johne's disease (JD) is very important for the control of the disease in dairy herds (Stabel *et al.,* 2002). Many publications considered the PCR assay as an alternative rapid and powerful tool to detect DNA of *Map* directly in faecal samples (Behr and Collins, 2010). IS900- PCR assay was reported to be highly specific and sensitive diagnostic test; this supports its potential value for rapid and effective diagnosis of

Johne's disease (Tripathi *et al.,* 2006). Also, Poupart *et al*. (1993) reported the *Map* F57 segment to be highly specific and sensitive target for detection of *Map* by PCR assay. So, the aim of this study was to evaluate both the IS900 and F57 segments as a target for detection of *Map* DNA in clinical samples using conventional PCR and IS900 real-time PCR assays for diagnosis of Johne's disease in dairy cattle.

At first, the relative sensitivities (limits of detection) of different PCR assays were determined by amplification of 10-fold serial dilutions of pure DNA of K10 strain of *Map* starting from 1.1µg/µl to 10^{-15}µg/µl which corresponded to 220 genomic copies of DNA to 2^{-12} genomic copy. As shown in figures 1 and 2, the detection limits of conventional PCR assay using IS900 and F57 primer sets were $1.1x10^{-6}$ µg/µl and $1.1x10^{-12}$ µg/µl, respectively. Ikonomopoulos *et al.* (2004) previously reported the sensitivity of the IS900 PCR assay to be 1500 genome copies (7.5 pg DNA) in 10-fold serial dilutions of K10 strain of *Map*. Since IS900 repetitive element occurs with 14-20 copies in the *Map* genome (Bull *et al*., 2000; Enosawa *et al.,* 2003; Li *et al.,* 2005), it was presumed that PCR with this target region would be more sensitive than other PCR based on other non-repetitive target (Collins *et al*., 1989; Green *et al.,* 1989). In the present study, per contra, the sensitivity of PCR assay targeting the F57 locus with one copy (Vansnick *et al.,* 2004) was higher than that of IS900 primer set for detection of pure DNA of K10 strain. Almost similar results were reported by Mobius *et al.* (2008) who found no differences in the analytical sensitivities of single PCR targeting ISMav2 loci which occur in three copies, F57 with one copy and locus 255 with one copy. Also, Vansnick *et al*. (2004) found no difference in the sensitivity of PCR targeting F57 and IS900 loci. This unexpected difference in the sensitivity can be attributed to differences in the binding abilities of different primer sets and PCR conditions that may have a higher influence on the analytical sensitivity of the PCR than the number of copies of the specific target region in the genome as reported by Mobius *et al.* (2008).

On the other hand, using real-time PCR utilizing the IS900 primer set, the detection limit was found to be $1.1x10^{-8}$ µg/µl of pure DNA which corresponds to $2.2x10^{-6}$ genomic copy (Fig. 3). These results indicated that the real-time PCR assay was more sensitive than conventional PCR. Rodriguez-lázaro *et al.* (2005) reported the detection limit of real-time PCR utilizing the same primers to be 1 genomic DNA molecule per reaction.

The second aim of the study was to apply and compare the evaluated PCR assays for direct detection of *Map* DNA in different clinical samples including both faecal and milk samples. Methods of DNA extraction have a significant effect on the sensitivity of nucleic acid based tests. Therefore, the same application of PCR can have many differences in the results obtained depending on the used DNA extraction method (Christopher-Hennings *et al*., 2003). So, in this study, we used a crude method for DNA extraction from a large volume of faecal samples which was previously reported by Stable *et al*. (2004). It was based on a rapid boiling procedure without the need for special equipment. This method was recorded to be the most sensitive and less expensive method and can be used for preparing 50 faecal samples to be screened for *Map* by PCR in only one day work (Collins, 1996). Figures 4, 5 and 6 showed the PCR results of representative positive and negative faecal samples using IS900 and F57 conventional PCR assays and IS900 real-time PCR, respectively.

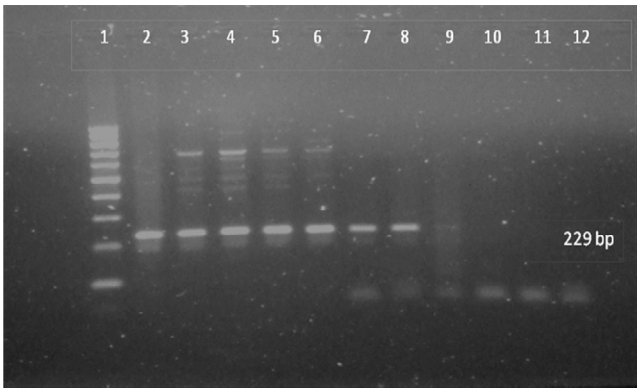

Fig.1 Detection limit of *Map* DNA by conventional PCR using IS900 primer set. Lane 1: 100bp ladder DNA marker; lanes 2 to 11: 10-fold serial dilutions of DNA of *Map* reference strain (K10) starting from 1.1µg/µl until $1.1x10^{-7}$ µg/µl. Lane 12: negative control.

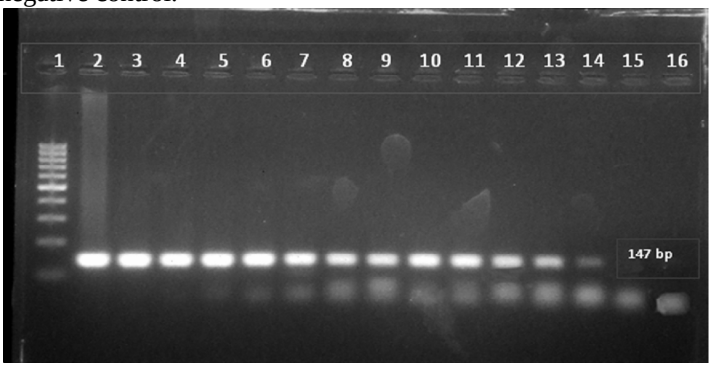

Fig.2 Detection limit of *Map* DNA by conventional PCR using F57 primer set. Lane 1: 100bp ladder DNA marker; lanes 2 to 15: 10-fold serial dilutions of DNA of *Map* reference strain (K10) starting from 1.1µg/µl until $1.1x10^{-13}$ µg/µl. Lane 16: negative control.

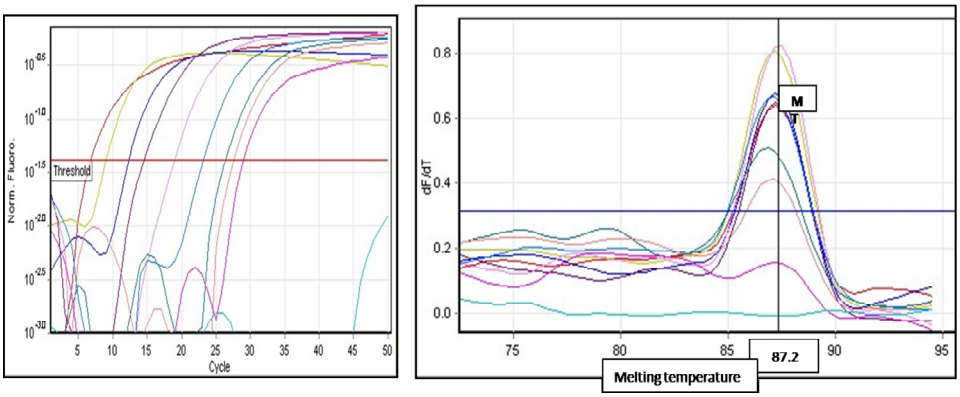

Fig.3 Quantitative and melting curves of the sensitivity of IS900 Real-Time PCR for 10-fold serial dilutions of DNA of *Map* reference strain (K10) starting from 1.1µg/µl until $1.1x10^{13}$ µg/µl.

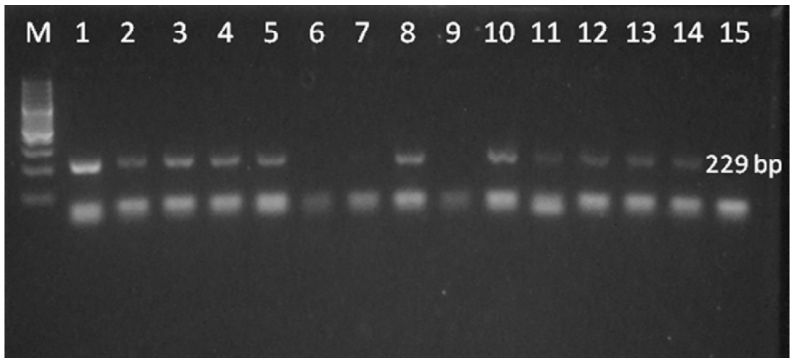

Fig.4 Detection of *Map* DNA in faecal samples using IS900 conventional PCR producing 229bp band. Lane M: 100-bp ladder DNA marker; lane 1: positive control (strain K10 DNA), lanes 2-5, 8, 10-14: positive samples, lanes 6, 7, 9: negative samples, and lane 15: negative control.

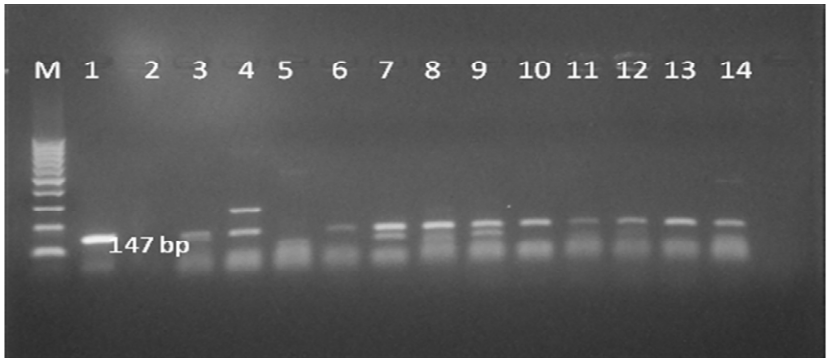

Fig.5 Detection of *Map* DNA in faecal samples using F57 conventional PCR producing 147bp band. Lane M: 100-bp ladder DNA marker; lane 1 positive control (strain K10 DNA), lanes 3, 4, 6-14 are positive samples; lane 5: negative samples and lane 2: negative control.

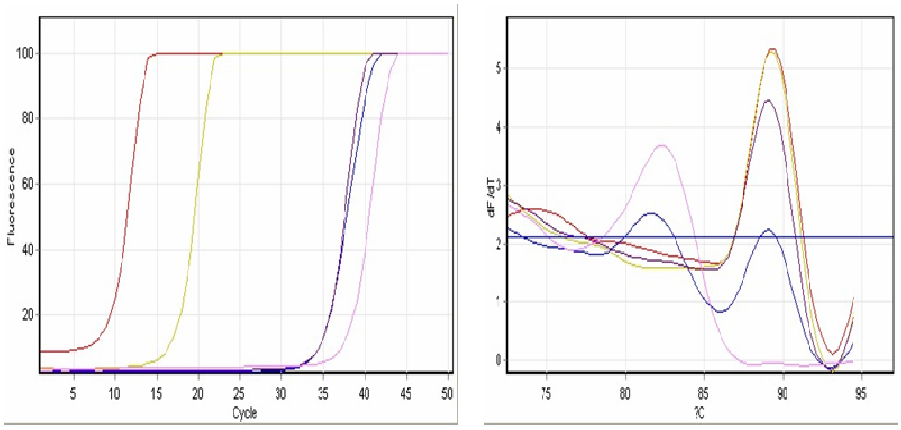

Fig.6 Quantitative and melting curves for the detection of *Map* DNA in faecal samples by using IS900 Real-Time PCR assay.

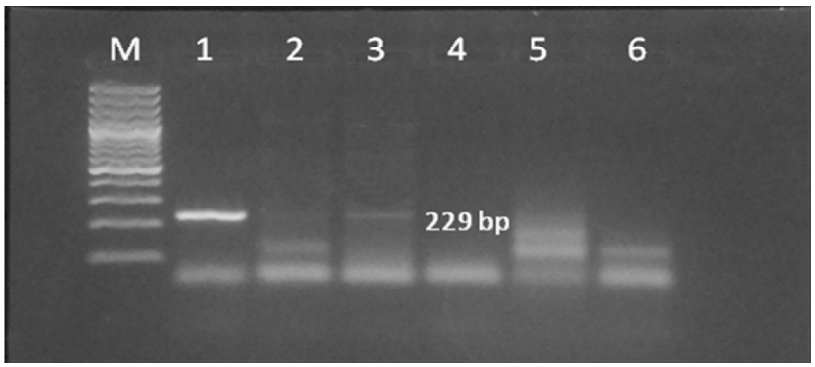

Fig.7 Detection of *Map* DNA in milk samples using IS900 conventional PCR producing 229bp band. Lane M: 100-bp ladder DNA marker; lane 1: positive control (strain K10 DNA), lanes 2, 4, 5: negative samples; lane 3: positive sample and lane 6: negative control.

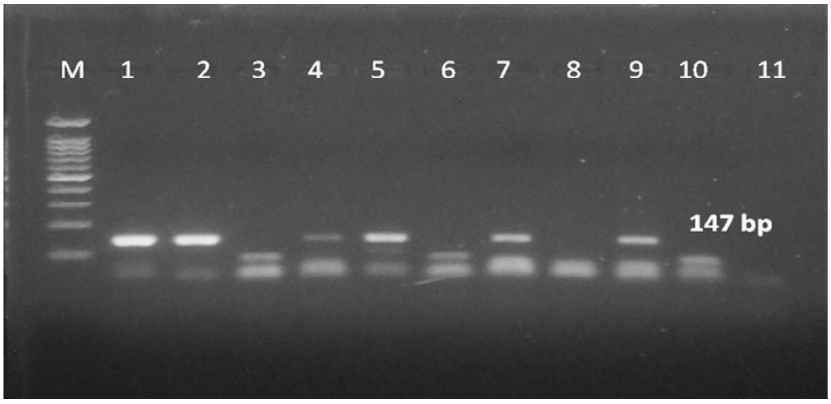

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Fig.8 Detection of *Map* DNA in milk samples using F57 conventional PCR producing 147bp band. Lane M: 100-bp ladder DNA marker; lane 1: positive control (strain K10 DNA), lanes2, 4, 5, 7, 9: positive samples; lanes 3, 6, 8 and 10: negative samples, and lane 11: negative control.

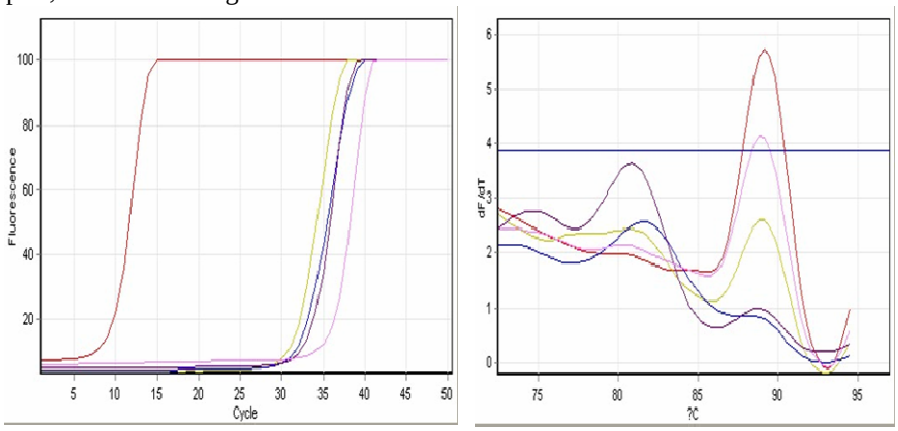

Fig.9 Quantitative and melting curves for the detection of *Map* DNA in milk samples by using IS900 Real-Time PCR assay.

Table.1 Number and percentages of *Map* positive faecal and milk samples collected from diarrheic and apparently healthy dairy cows using different PCR assays

Animal	Technique	Faecal samples		Milk samples		Total
		Positive No.	%	Positive No.	%	
Diarrheic dairy cows	IS900 conventional PCR	27^{aA}	35.06%	4^{aB}	5.2%	77
	F57 conventional PCR	25^{aA}	32.47%	4^{aB}	5.2%	77
	IS900 real-time PCR	31^{aA}	40.25%	5^{aB}	6.5%	77
Apparently healthy dairy cows	IS900 conventional PCR	20^{aA}	27.4%	3^{aB}	4.1%	73
	F57 conventional PCR	19^{aA}	26.03%	1^{aB}	1.4%	73
	IS900 real-time PCR	33^{bA}	45.21%	3^{aB}	4.1%	73

Values with different superscript in the same column mean significant difference (p<0.005).
Values with different superscript in the same row mean significant difference (p<0.001).

As shown in table "1", out of 150 faecal samples, 47 (31.33%), 44 (29.33%), 64(42.66%) were found positive by conventional IS900 PCR, conventional F57 PCR and Real-time PCR assay utilizing the IS900 primer set, respectively. Statistical analysis showed that there is no significant difference between conventional PCR assays using the two primer sets (IS900 and F57) while there was a highly significant difference (p<0.005) between conventional and real-time PCR assays which declared the real-time PCR to be more sensitive in the direct detection of *Map* in faecal samples than the conventional PCR. The prevalence of *Map* as determined by different assays was slightly higher than that of Amin *et al.* (2011) who reported the prevalence rate of *Map*-infected cows to be 22.5% by the IS900 PCR assay and that of Salem *et al.* (2005) who found 38% positive samples from total 200 animals and very higher than that of El-Sawy *et al.* (2011) who found only 14.67% positive samples from total 109 samples. This difference may be attributed to difference in method of detection, the number of samples, the environmental conditions of the farms and the animal age.

The data in table "1" also showed the number and percentages of positive samples among both diarrheic dairy cows and apparently healthy dairy cows. Among the diarrheic dairy cows samples, *Map* DNA was detected in 27 (35.06%), 25 (32.47%) and 31 (40.25%) samples using IS900PCR, F57 PCR and IS900 Real-time PCR assays, respectively. There was no significance difference between the ability of different PCR assays for detection of *Map* DNA in samples collected from diarrheic dairy cows. This can be explained by the fact that diarrheic dairy cows which are in the clinical stage of the disease-shed a large number of mycobacteria in faecas and so can be detected simultaneously by all the used PCR assays (Salem *et al.,* 2005). Among the examined apparently healthy dairy cows samples, PCR detected *Map* DNA in 20 (27.4%) and 19 (26.03%) samples using IS900 primer set and F57 primer set, respectively. On the other hand, the real-time PCR assay utilizing the IS900 primer set was able to detect *Map* DNA in 33 (45.21%) of apparently healthy dairy cows. There was a significance difference between the results of real-time PCR assay versus other conventional PCR assays (p<0.005). This could be attributed to the fact that apparently healthy dairy cows shed

only few numbers of mycobacteria in the faecas which can be better detected using real-time PCR assay because of the sensitivity of the SYBR green florescence for detection of minute amount of double stranded PCR product (Cocolin *et al*., 2011). Considering milk samples, *Map* DNA was successfully detected in 7 (4.67%), 5 (3.33%) and 8 (5.33%) samples by IS900 conventional PCR, F57 conventional PCR and IS900 real-time PCR assays, respectively. Figures 7, 8 and 9 showed the PCR results of representative positive and negative milk samples using IS900 and F57 conventional PCR and IS900 real-time PCR, respectively. Statistically, there was no significant difference between the three assays in *Map* detection in milk samples. This may be explained by the nature of the milk as a difficult specimen for the detection of organisms by PCR where it contains large amounts of PCR inhibitors as fat and calcium ions (Lantz *et al.*, 1994; Bickley *et al.*, 1996). Also, Odumeru *et al*. (2001) reported that apart from the low number of organisms in milk sample, it is also difficult to lyzes the *Map* present in milk by most of methods used for DNA extraction from milk.

The data in table "1" also showed the number and percentages of positive milk samples among both diarrheic and apparently healthy dairy cows. Among the diarrheic dairy cows samples, *Map* DNA was detected in 4 (5.2%), 4 (5.2%) and 5 (6.5%) milk samples using IS900 PCR, F57 PCR and IS900 Real-time PCR assays, respectively. There was no significant difference between the ability of different PCR assays for detection of *Map* DNA in milk samples collected from diarrheic dairy cows. Among the examined apparently healthy dairy cows samples, PCR detected *Map* DNA in 3 (4.1%) and 1 (1.4%) milk samples using IS900 primer set and F57 primer set, respectively. On the other hand, the real-time PCR assay-utilizing the IS900 primer set was able to detect *Map* DNA in 3 (4.1%) milk of apparently healthy animals. There was no significant difference between the results of real-time PCR assay versus other conventional PCR assays ($p<0.005$).

Comparison between both faecas and milk as samples used for detection of *Map* DNA using different PCR assays is shown in table "1". Statistical analysis using Chi-square shows a significant difference between both samples ($p < 0.001$). This confirms the superiority of faecal samples versus milk samples as the optimum sample for diagnosis of *Map* by PCR assay. Sweeny *et al*. (1992) documented that cows with clinical disease or asymptomatic cows with heavy faecal shedding may shed less than 1 CFU/ml of milk. They also demonstrated that asymptomatic cows infected with *Map* could potentially transmit paratuberculosis *via* milk. Although the concentration of the organisms in the milk was found to be low (2-8 CFU/ ml), the risk of infection would be multiplied due to the large quantity of milk consumed by a calf (4 litres /day). Moreover, Gao *et al*. (2009) reported that there was no significant correlation between the number of CFU cultured from milk and faecas. The found that the animals with high numbers of CFU in milk culture may not be detected by faecal culture at all, and vice versa. This suggests that shedding of *Map* in faecas and milk is not synchronized. These authors also mentioned that to identify suspected Johne's-infected animals, it would be better to use both milk and faecas simultaneously to enhance the diagnostic rate.

In conclusion, real-time PCR was significantly more sensitive in direct detection of *Map* DNA in cattle faecal samples than conventional PCR assays.

Also, comparison between IS900 and F57 primer sets showed that there was no significant difference between the two primers in direct detection of *Map* in clinical samples. Faecal samples were confirmed to be the optimum samples versus milk samples for diagnosis of *Map* by different PCR assay.

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