Dairy herd prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in bulk-tank milk samples obtained from three regions in Fars Province, Iran by nested PCR

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**ABSTRACT**
A cross-sectional study was conducted from March through August 2006 in dairy herds in Fars province, southern Iran to determine the herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection and associated risk factors. Bulk-tank milk samples and management information were collected from 110 dairy herds in the province. Among study populations, 12 herds (11%, 95% CI: 5-17%) were positive for MAP infection based on IS900 nested PCR. Statistical analysis using multivariable logistic regression showed that contamination of udders of periparturient cows with manure (OR=6.4, P=0.02) and history of suspected cases of Johne's disease in the herd (OR=6.7, P=0.04) were significantly associated with herd infection status. No relationship between breed, herd size and other management practices was found in this study. It is recommended to conduct further epidemiologic studies to determine cow-level prevalence and risk factors, and to evaluate the economic consequences of the MAP infection in the region.

**INTRODUCTION**
Johne's disease is a chronic disease of ruminants worldwide caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The Johne's disease in the dairy community has long been considered as an economically-important disease by reports of the negative impact on milk production and the overall economic health of dairies (Johnson et al., 2001; Beaudeau et al., 2007).

Diagnostic tests such as ELISA, agar gel immunodiffusion (AGID) test and fecal culture are being used commonly. Also PCR methods, targeting MAP specific insertion sequence (IS900) or other species-specific genes have been developed to increase sensitivity and specificity of diagnosis. On the other hand, presence of the pathogen in milk has been confirmed by several researchers (Sweeney et al., 1992; Grant, 2003). It was shown that MAP could be detected directly from quarter milk and bulk-tank milk by IS900 PCR (Pillai and Jayarao, 2002; Buergelt and Williams, 2004). Therefore, application of PCR for milk samples from individual cows or at herd level as bulk-tank milk have proven useful for prevalence estimation of infection with MAP by some studies (Corti and Stephan, 2002; Stabel et al., 2002).

The aim of the present study was to estimate the current herd-level prevalence of MAP infection in dairies located in the Fars province, southern Iran using IS900 nested PCR on bulk-tank milk samples, and to identify possible associated risk factors.

**MATERIALS AND METHODS**

*Study population*
A cross-sectional study was conducted in the Fars province, southern Iran to determine the prevalence of MAP infected-herd in the region. Overall 110 herds were included in the study. A structured questionnaire was used to collect data about general herd information such as herd size and breed as well as information about possible risk factors for MAP infection. Most factors were compiled from the literature.

*Extraction of DNA and PCR process*
Milk samples were collected from well mixed bulk-tank of study herds and brought cooled to the laboratory. For detection of MAP, 50 ml was stored at -20°C up to time of investigation.
Extraction of DNA was undertaken as described by Stabel et al. (2002) with minor modifications.

**IS900** nested PCR was conducted as described by Corti and Stephan with minor modification (Corti and Stephan, 2002). For samples with 413 bp band and suspected samples, 3 µl from the primary amplification were used for nested PCR. The primers are shown in Table 1.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P90</td>
<td>5′-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3′</td>
</tr>
<tr>
<td>P91</td>
<td>5′-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3′</td>
</tr>
<tr>
<td>AV1</td>
<td>5′-ATG TGG TTG CTG TGT TGG ATG G-3′</td>
</tr>
<tr>
<td>AV2</td>
<td>5′-CCG CCG CAA TCA ACT CCA G-3′</td>
</tr>
</tbody>
</table>

**Statistical analysis**

Confidence limits (95%) for prevalence were calculated using standard normal distribution. The unit of statistical analysis was herd. To evaluate associations between suggested risk factors and herd level status for MAP infection (outcome variable), univariable and multivariable logistic regression analysis was done. Factors with P-value <0.2 in the univariable analysis were included in the multivariable logistic regression analysis.

Multicollinearity amongst putative risk factor variables was assessed using Spearman Rank correlation coefficients. All computed coefficients were less than 0.40. Also correlation between each independent variable and linear combination of other variables was investigated using variance inflation factor (VIF). All VIF were less than 1.2, revealing lack of significant collinearity in the dataset. The multivariable logistic regression model was constructed using a step-wise backward elimination approach based on significant change in -2 log-likelihood. Those variables with Wald statistic values with P<0.05 in the final model were considered as statistically significant. To account for clustering of herds in each district, location of herds (area) was forced to remain in the model and presence of two-way interaction between location and the other included variables was investigated. All statistical analysis was conducted using SPSS software (Version 11.5).

**RESULTS**

A single band of 298 bp for each of the positive milk sample was detected by PCR amplification of the MAP specific insertion sequence **IS900** and subsequent agarose gel.
analysis of the amplified products (Fig 1). Among 110 bulk-tank milk samples, 12 (11%, 95% CI: 5-17%) were positive in IS900 nested PCR.

The prevalence of positive milk samples in the three district of Fars province were different ranging from 8.6 to 23% which was not significant ($\chi^2=3.3$, df=2, P=0.19).

With univariable analysis six factors were selected based on P value <0.2 (Table 2) and included in the multivariable step-wise logistic regression analysis. In the final logistic model, two out of six factors were significant at the 0.05 level. Location of herds was retained in the model although it was not significant. No significant interaction was detected in the regression model. The Wald statistic, standard error, odds ratios and the 95% confidence level for the factors are provided in Table 2. Herds with low sanitation of periparturient cows as measured by high contamination of udders with manure had 6.4 times the odds of being positive for MAP infection. Also herds with history of suspected cases of Johnne's disease had 6.7 times more likely to be positive by PCR than herds without such history.

**Table 2.** Multivariable logistic regression analysis of risk factors for *Mycobacterium paratuberculosis* infection in 110 dairy herds in the Fars province, southern Iran based on IS900-PCR in bulk-tank milk samples

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Wald statistic</th>
<th>SE</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contamination of udders with manure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5.18</td>
<td>0.81</td>
<td>0.02</td>
<td>6.38</td>
<td>1.29-31.49</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>History of Johnne's disease in the herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.91</td>
<td>0.96</td>
<td>0.04</td>
<td>6.70</td>
<td>1.02-44.16</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
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</table>

**DISCUSSION**

The overall herd-level prevalence of MAP in dairy herds investigated was approximately 11% based on IS900 nested PCR on bulk-tank milk samples. History of suspected cases of Johnne's disease and low sanitation of periparturient cows as measured by contamination of udders with manure, both indicate a higher likelihood that a herd is infected with MAP infection. It is critical to conduct in-depth epidemiologic studies to identify cow-level prevalence and economic consequences of MAP infection throughout the country.

Though several potential risk factors were not significant in the present study, as suggested by Berghaus et al. (2005), this does not necessarily imply that they are not important. Considering the various environmental, nutritional and management conditions over the world, some factors may work as important causal complement for MAP infection in one region and might not be so important in another region.

**ACKNOWLEDGMENTS**

The authors would like to thank Professor Leonardo Sechi, Department of Biomedical Sciences, University of Sassari, Italy for providing the MAP for positive control in PCR. This study was supported by Grant No. 84-VE-1780_C308 Shiraz University, Iran.

**REFERENCES**


