

PD 03 Real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples

C.R. Dubosson^a, C. Conzelmann^b, W. Zimmermann^c, H. Hänni^b, R. Miserez^a, P. Boerlin^a, J. Frey^a and P. Kuhnert^a

^aInstitute of Veterinary Bacteriology, ^bInstitute for Animal Pathology, ^cDept. of Clinical Veterinary Science
University of Bern, Laenggass-Strasse 122, CH-3001 Bern, Switzerland, Tel. 0041-31-6312485, Fax 0041-31-6312634
peter.kuhnert@vbi.unibe.ch

Summary: Two real-time PCR assays for detection of *Mycoplasma hyopneumoniae* (*Mhyop*) in clinical lung samples were established and validated in parallel. One is targeting a repetitive DNA element (REP assay) the other a putative ABC transporter gene (ABC assay). The two assays were shown to be 100% specific when testing pig lungs from defined negative farms. When investigating defined positive farms the REP assay tested with a sensitivity of about 50%, the ABC assay with 90%. The two assays together, however detected 100% of positive farms. Within a single positive farm on average 90% of the samples tested positive with the REP or ABC assay. Analysing a set of 41 lungs from infected pigs from routine diagnostic the REP assay detected 50% and the ABC assay 70%, while both assays together had a sensitivity of 85%.

Keywords: enzootic pneumonia, pig disease, diagnostic, TaqMan, eradication

Introduction: The current diagnosis of enzootic pneumonia (EP) of swine is based on a polyphasic approach integrating various aspects of the disease. These include clinical signs, serology, pathological investigation of lungs and detection of the pathogen *Mhyop*. The latter can be done by immunofluorescence, by Giemsa staining or by culture. The PCR technology and particularly the real-time PCR (rtPCR) would be the method of choice for pathogen detection, since some unspecific cross-reaction with other mycoplasmas was observed with immunofluorescence, Giemsa staining is of low specificity and culture is fastidious and time consuming. Several PCR assays have been developed on specific *Mhyop* DNA fragments, including two nested-PCR (Stärk et al., 1998; Verdin et al., 2000). Nevertheless, this kind of assay is not convenient for routine diagnosis of EP. Therefore, we developed and validated two rtPCR methods for the routine detection of *Mhyop* in clinical lung samples.

Materials and Methods: A total of 208 lungs obtained from slaughterhouses were analysed in this study. After macroscopic assessment and sampling for histological analysis lungs were forwarded for further bacteriological examination. In order to have defined positive and negative sample groups and to analyse several samples from a defined case, we selected 16 farms with clearly defined sanitary status regarding EP (11 EP-positive and 5 EP-negative farms with normally 10 lungs/farm). The farm status definition was based on the presence or absence of clinical signs (mainly chronic, non-productive coughing), epidemiological tracing of the farm, typical necropsy and histopathological lesions and positive immunofluorescence results at the farm level, which together represent a so called "mosaic diagnosis". In addition, 70 lungs were from routine EP diagnosis and were selected by pathologists according to clinical and macroscopic signs. These single lungs were collected out of several typical lungs of animals from the same farm. Bronchial swabs were taken and a lysate was prepared for PCR detection of *Mhyop*. DNA sequencing of part of the two target genes was done from several *Mhyop* probes. The REP assay targeting a repetitive DNA element is using a classical TaqMan[®] probe to detect and specify the amplicon. The ABC assay targeting a putative ABC transporter gene uses a shorter TaqMan[®] MGB probe. The rtPCR were established using several bacterial and mollicutes DNA before they were validated on clinical lung material.

Results: Sequence variations in both target genes were observed and considered when choosing primers and probes. Both rtPCR assays tested positive with *Mhyop* strains used and were always

negative with DNA from other mollicutes and bacteria commonly isolated from pig. Within the EP-negative set consisting of 44 samples from 5 farms both rtPCR tested negative for all samples (100% specificity). For the EP-positive set 94 samples obtained from 11 farms were tested. The REP assay tested positive on 44 samples out of 6 farms (sensitivity of 48.35 +/-10.27%, $p<0.05$). Within these REP positive farms the assay tested positive between 80-100% (mean 93.33%) of the lungs originating from the same herd. Five farms were negative in the REP assay for all individual lung samples tested. The ABC assay tested positive on 77 samples from 10 farms (sensitivity of 82.80 +/-7.67%, $p<0.05$). Within these ABC positive farms the assay tested positive between 60-100% (mean 95.00%). The assay tested negative for all 10 samples collected from 1 farm. The two rtPCR assays together showed positive for all the 11 positive farms and the results within a positive farm were clearly positive (on average >90% of the individual samples). Five farms were negative with the REP assay but positive with the ABC assay. On the other side the single farm testing negative with the ABC assay gave 100% positive results with the REP assay. For further evaluation of the two rtPCR 70 single routine diagnostic cases were collected and assayed by rtPCR. These samples were retrospectively defined as consisting of 41 EP-positive and 29 EP-negative samples by the classical "mosaic diagnosis". For the 29 negative samples both the REP and ABC assays tested negative (100% specificity). Out of the 41 EP-positive diagnostic samples 21 were positive in the REP assay (sensitivity of 51.22 +/-15.30%, $p<0.05$). 12 REP-negative samples were positive in the ABC assay. The ABC assay tested positive on 28 samples (sensitivity of 68.29 +/-14.24%, $p<0.05$). 5 ABC-negative samples were positive in REP. The calculated sensitivity obtained by the use of both rtPCR assays in parallel was 84.52%.

Discussion: The rtPCR assays developed and validated are a valuable tool for diagnosis of EP, what is especially important in the light of a solid support for decisions being taken for the eradication of the disease. The assay is very specific and sensitive on the herd level. Analyzing single probes from suspicious herds as done in routine diagnosis might be prone to false negative results, as the findings with the diagnostic samples indicate. We recommend to take at least lungs from 3 individuals in such cases. PCR detection as a method is less dependent on subjective interpretation compared to the immunofluorescence or pathological examinations. This together with the speed and large sample numbers that can be tested by rtPCR makes it the method of choice, moreover since it is less sensible to contamination. In order to increase the overall sensitivity of the method it would be useful to develop a multiplex assay, which allows to carry out both the REP and ABC assay in a single tube.

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