Development and evaluation of a real-time quantitative PCR assay for Culicoides imicola, one of the main vectors of Bluetongue (BT) and African Horse Sickness (AHS) in Africa and Europe

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INTRODUCTION

Bluetongue (BT) and African horse sickness (AHS) are infectious arthropod-borne viral diseases caused by viruses belonging to the genus Orbivirus within the family Reoviridae and transmitted by certain species of biting midges belonging to the genus Culicoides (Diptera: Ceratopogonidae). 32 species among the 1340 species of the genus Culicoides have been connected with BT transmission (Meiswinkel et al., 2004; Meillon, 1990). The explosive outbreaks of BTV in the Mediterranean Basin since 1998 have been fuelled largely by the classical Afro-Asian vector C. imicola. As outbreaks have been reported in the Eastern Mediterranean Basin (Bulgaria, Serbia, Kosovo, Cinadl, Montenegro, northern Greece, Bosnia Herzegovina) and in northern Europe (Belgium, Germany, Ireland and France) where the classical Afro-Asian vector C. imicola has never been detected during insect surveys (Baylis and Mollor 2001). Thus, the tool of study, based on previous studies on the development of diagnostic tools (PCR and phylogenetic analysis) was to develop a reproducible real-time PCR method that targets the first internal transcribed spacer (ITS-1) of the ribosomal DNA of C. imicola as developed. This real-time PCR assay was first performed on 10-fold serial dilutions of purified plasmid DNA containing specific C. imicola as ITS-1. It was then possible to construct standard curves with a large correlation coefficient (r = 0.98) in the range of 10^(-4) to 10^(-10) copies of purified DNA. The performance of this PCR assay was evaluated in comparison with the traditional microscopy method on Culicoides trapped along the Mediterranean coastal mainland France, ROC statistical analysis was carried out using morphology as gold standard and the area under the ROC curve had a satisfactory value of 0.975. The results indicated that this real-time PCR assay holds promise for monitoring C. imicola as population in both surveillance and research programs because of its good specificity (92%) and sensitivity (95%).

MATERIALS AND METHODS

Field collections

The sites at risk selected for the entomosurveillance of C. imicola as into mainland France are spread at 50 km intervals along the Mediterranean coast (Baldet et al., 2004) with one collection per month from April to November 2004. Specimens of the genus Culicoides were identified at species level and the absence of C. imicola was confirmed on the basis of the collection spot. A total of 8292 Culicoides were collected in 99 light traps set in two sites at risk in the south of France, considered as gold standard. These trap catches were reconstituted with 10, 50, 100 or 1000 C. imicola as described (3 trap catches for each condition).

Extraction of genomic DNA and construction of C. imicola as ITS-1 plasmid

After grinding up to 50 mg of insects in a mortar and pestle containing liquid nitrogen, the extraction of DNA was performed with the MOCUS system (Qiagen, Germany) using the «DNEasy Tissue kit» (Qiagen, USA). The purified ITS1 of C. imicola as was cloned into PCR-Blunt vector (Zero Blunt PCR Cloning Kit, Invitrogen, USA). At least of this plasmid, stored at -80°C was used for the establishment of a reproducible standard curve run in each plate.

C. imicola as ITS-1 quantitative PCR amplification

Amplification of the ITS-1 sequence of C. imicola as performed with a SYBR Green detection method in a total volume of 20 ml consisting of 2x PCR reaction buffer (Stratagene, France), 300 nM of each primer (PanCulR-5’-TTCATATGGGATTGCTTTCTATCC-3’; PanCul detection assay (Diptera: Ceratopogonidae). 32 species among the 1340 species of the genus Culicoides (Diptera: Ceratopogonidae). 32 species among the 1340 species of the genus Culicoides have been connected with BT transmission (Meiswinkel et al., 2004; Meillon, 1990). The explosive outbreaks of BTV in the Mediterranean Basin since 1998 have been fuelled largely by the classical Afro-Asian vector C. imicola. As outbreaks have been reported in the Eastern Mediterranean Basin (Bulgaria, Serbia, Kosovo, Cinadl, Montenegro, northern Greece, Bosnia Herzegovina) and in northern Europe (Belgium, Germany, Ireland and France) where the classical Afro-Asian vector C. imicola has never been detected during insect surveys (Baylis and Mollor 2001). Thus, the tool of study, based on previous studies on the development of diagnostic tools (PCR and phylogenetic analysis) was to develop a reproducible real-time PCR method that targets the first internal transcribed spacer (ITS-1) of the ribosomal DNA of C. imicola as developed. This real-time PCR assay was first performed on 10-fold serial dilutions of purified plasmid DNA containing specific C. imicola as ITS-1. It was then possible to construct standard curves with a large correlation coefficient (r = 0.98) in the range of 10^(-4) to 10^(-10) copies of purified DNA. The performance of this PCR assay was evaluated in comparison with the traditional microscopy method on Culicoides trapped along the Mediterranean coastal mainland France, ROC statistical analysis was carried out using morphology as gold standard and the area under the ROC curve had a satisfactory value of 0.975. The results indicated that this real-time PCR assay holds promise for monitoring C. imicola as population in both surveillance and research programs because of its good specificity (92%) and sensitivity (95%).

Sensitivity and specificity

The sensitivity and specificity of the real-time PCR assay were performed with the DNeasy Tissue kit (Qiagen, USA). The purified ITS1 of C. imicola as was cloned into PCR-Blunt vector (Zero Blunt PCR Cloning Kit, Invitrogen, USA). At least of this plasmid, stored at -80°C was used for the establishment of a reproducible standard curve run in each plate.

Conventional cytochrome oxidase specific and C. imicola as specific PCR amplification

To control the quality of DNA extracted from the trap catches, a conventional PCR performed on C. imicola as specific cytochrome oxidase (COX) was designed (Cox-F 5’-CAGAAATATATATAACTCAGTGC-3’; Cox-R 5’-GGAAGTGGATTGAATTTG-3’). In addition, a conventional PCR specific of C. imicola as ITS-1 was included to assess the performances of the real-time PCR assay PanCul/PanCulR-PCR for the C. imicola as (Cetresossah et al., 2004).

Data analysis

A receiver operating characteristic (ROC) analysis was performed to assess the performances of the C. imicola as real-time PCR test that could be considered as a diagnostic and surveillance tool. In our study, the true identification of C. imicola as was performed by a microscopy examination and was considered as our gold standard. All statistical analyses were carried out with the statistical software (Statistica 6), sensitivity and specificity were estimated using “cutoff” data command. Ninety-five percent confidence intervals (95% CI) were provided assuming a binomial distribution.

RESULTS AND DISCUSSION

Linearity of the real-time PCR method

The standard curve was made over the entire quantification range and resulted in an error of less than 1.0 with a linear regression value R² of 0.999, indicating a strong correlation between the ranges used from 10-1 to 10^10 copies (Figure 1).

Sensitivity and specificity

The quantification of the real-time PCR test was evaluated by testing DNA extracted from 100 light trap catches collected in Southern France, considered as gold standard where no specimens of C. imicola as were detected except for one trap catch with one single specimen of C. imicola as. Either 1 or 5 specimens of C. imicola as were randomly added in some of the 99 sampled trap catches. To assess the capacity of the real-time PCR assay to quantify C. imicola as, 12 individual trap catches were reconstituted with 10, 50, 100 or 1000 C. imicola as previously described (3 trap catches for each condition).

The ROC analysis was carried out using C. imicola as as area under curve was 0.9752 (between 0.7634 and 0.9721 with a 95% confidence interval, CI). The cut-off point to which sensitivity and specificity were optimal was for a C. imicola as value of 30.5. The sensitivity of the test for this cut-off was 95% (95% CI: 0.854-1.0) and the specificity was 92% (95% CI: 0.824-0.975).

CONCLUSION

The choice of the morphological determination as a reference test is justified even if both PCR methods (conventional and real-time) are concordant and gave similar levels of sensitivity (respectively 97.5% and 95%) and specificity (respectively 96% and 92%) with equivalent concordance rate (0.97 versus 0.93). The difference in C. imicola as CI values in samples containing 1 specimen or samples containing 5 specimens of C. imicola as was not possible. A corresponding C. imicola as value of 30.5 was chosen and therefore gave a relatively high sensitivity of 95% and a specificity of 92%. 

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REFERENCES