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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The current microscopy method for identifying the Culicoides imicola Kieffer, 1913 species can be time and labour intensive. There is a need for the development of a rapid and quantitative tool to quantify the biting midges Culicoides imicola ss in light-trap catches. A reproducible and sensitive real-time polymerase chain reaction method that targets the internal transcribed spacer (ITS-1) of ribosomal DNA of C. imicola ss species was developed. This real-time PCR assay was first performed on 10-fold serial dilutions of purified plasmid DNA containing specific C. imicola ss ITS-1. It was then possible to construct standard curves with a high correlation coefficient (r^2 = 0.99) in the range of 10^{-2} to 10^{-8} ng of purified DNA. The performances of this PCR were evaluated in comparison with morphological determination 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 0.9752. The results indicated that this real-time PCR assay holds promise for monitoring C. imicola ss population in both surveillance and research programmes because of its good specificity (92%) and sensitivity (95%).

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* Latreille have been connected with BTV transmission (Meiswinkel et al., 2004; Mellor, 1990). The explosive outbreaks of BTV in the Mediterranean Basin since 1998 have been fuelled largely by the classical Afro-Asian vector *C. imicola ss*. However, outbreaks have been reported in the Eastern Mediterranean Basin (Bulgaria, Serbia, Kosovo, Croatia, Montenegro, northern Greece, Bosnia Herzegovina) and in northern Europe (Belgium, Germany, Holland and France) where the classical Afro-Asian vector *C. imicola ss*, has never been detected during insect surveys (Baylis and Mellor 2001). Thus the aim of this study, based on previous studies on the development of diagnostic assays and phylogenetic analysis) was to develop a reproducible real-time PCR method that targets the first internal transcribed spacer (rDNA ITS-1) for the quantitative surveillance of *C. imicola ss* in light-trap catches and to evaluate the performances of this method in comparison with the morphological determination which is tedious and time consuming but which is considered as the current gold standard

MATERIALS AND METHODS

Field collections

The sites at risk selected for the entomosurveillance of *C. imicola ss* into mainland France are spread at 50 km intervals along the French Mediterranean coast (Baldet et al., 2004) with one collection/night/site/month from April to November 2004. Specimens of the genus *Culicoides* were identified at species level and the absence of *C. imicola ss* was confirmed based microscopically upon wing pattern (Delécolle et al., 1985) .Then, 99 original trap catches, coming from this entomosurveillance network, were reconstituted with specimens of all insects families, and specimens belonging to different species of *Culicoides*. One additional light trap catch was an original sample with already one specimen of *C. imicola ss*. Either 1 or 5 specimens of Corsican *C. imicola ss* were further added randomly in some of the 99 sampled trap catches To assess the capacity of the real-time PCR assay to quantify *C. imicola ss*, 12 individual trap catches were reconstituted with 10, 50, 100 or 1,000 *C. imicola ss* as previously described (3 trap catches for each condition).

Extraction of genomic DNA and construction of C. imicola ss 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 DNeasy Tissue kit (Qiagen, USA). The purified ITS1 *C. imicola ss* specific PCR product was cloned into PCR-Blunt vector (Zero Blunt PCR Cloning Kit, Invitrogen, USA). Aliquots of this plasmid, stored at -80°C were used for the establishment of a reproducible standard curve run in each plate.

C. imicola ss ITS-1 quantitative PCR amplification

Amplification was performed using the Mx3000P System with a SYBR Green I detection method in a total volume of 20 ml consisting of 2x PCR reaction buffer (Stratagene, France), 300 nM of each primer (PanCulR-5'-TGCGGTCTTCATCGAACCCAT-3'/Cul-imicola-5'-TTACAGTGGCTTCGGCAAG-3') under the following cycling conditions: an initial denaturation stage at 94°C for 5 min; then 40 cycles at [94°C, 1 min; 62°C, 1 min; 72°C, 30 sec] and a final dissociation phase. The plasmid PCR-Blunt-*C. imicola ss* ITS-1 construct was used in serial dilutions as a standard curve. Results were expressed in Ct values and the Ct values were determined at a preset threshold.

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

To control the quality of DNA extracted from the trap catches, a conventional PCR based on cytochrome oxidase was performed COX-F-5'-CAggTAAAATTAAAATATAAACTTCTGG-3'/COX-R-5'-GGAGGATTTGGAAATTGATTAGT-3') (Simon et al., 1994) In addition, a conventional PCR specific of *C. imicola ss* ITS-1 was included to assess the performances of the real-time PCR assay PanCul-R/Cul-imicola for the *C. imicola ss* PCR (Cêtre-Sossah et al., 2004)

Data analysis

A receiver operating characteristic (ROC) analysis was performed to assess the performances of the *C. imicola ss* real time PCR test that could be considered as a diagnostic and surveillance tool. In our study, the true identification of *Culicoides* was performed by a microscopy examination and was considered as our gold standard. All statistical analyses were carried out with intercooled Stata 8 software. Sensitivity and specificity were estimated by using "diagt" stata command. Ninety-five percent confidence intervals (CI 95%) were provided assuming a binomial distribution

RESULTS AND DISCUSSION

Linearity of the real time PCR method

The standard curve was linear over the entire quantification range and resulted in an error of less than 1.0 with a linear regression value r^2 of 0.999, indicating a strong correlation between the ranges used from 10^{-2} to 10^{-8} ng (Figure 1)

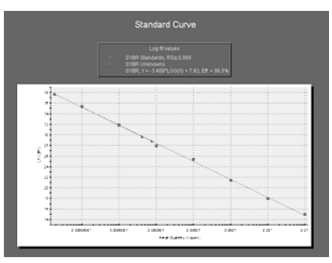


Figure 1: Linear regression of the plasmid PCR-Blunt-ITS-1 standard ranging from 1 fg to 10 pg was determined using fitpoints analysis method. The standard curve showed a regression coefficient r²=0.999, mean squared error, efficacy of 86.5% and slope = -3.408. Each point of the curve represents the average value (SEM) of three independent qPCR reactions. Figure 1 represents cycle numbers in y-axis and ng quantities in x-axis

Sensitivity and specificity

The quantitative real-time PCR method 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 ss* were detected except for one trap catch with one single specimen of *C. imicola ss*. Either 1 or 5 specimens of *C. imicola ss* were randomly added in 40 of these 99 samples. The distribution of the Ct values of negative versus positive samples in Figure 2 allowed the determination of a cut-off value at 30.5 (Figure 2)

Sensitivity and specificity

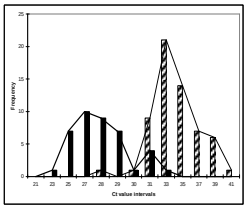


Figure 2: Distribution of the Ct value of 100 light trap catches samples from a French BT free area collected in 2004

		Morphology		
		Positive	Negative	Total
Real time PCR	Positive	39	5	44
	Negative	2	54	56
	Total	41	59	100

Table 1: Distribution of the results of the *C. imicola* ITS-1 screening real-time PCR versus morphological determination for one hundred light traps samples containing either 1 or 5 *C. imicola* within thousands of others insects

		Morphology		
		Positive	Negative	Total
Conventional PCR	Positive	40	2	42
	Negative	1	57	58
	Total	41	59	100

Table 2: Distribution of the results of the *C. imicola* ITS-1 conventional PCR versus morphological determination for one hundred light traps samples containing either 1 or 5 *C. imicola* within thousands of others insects

When correlating the Ct values obtained from the real-time PCR with the microscopy method, all 39 samples found positive by microscopy had Ct values between 22.5 and 30.5 while the 54 samples found negative by microscopy value were higher than 30.5.
As mentioned in Table 2, 39 out of 41 samples were positive by both the real-time PCR screening method and the microscopy whereas 54 samples were negative by both methods giving a concordance rate of 93% between microscopy and ITS-1 real-time PCR. The sensitivity and specificity of the real-time PCR were 95% and 92% respectively

These 100 samples were included in the ROC analysis (Figure 3), the 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 Ct value of 30.5. The sensitivity of the test for this cut-off was 95% (95% CI: 0.8824-1.0) and the specificity was 92% (95% CI: 0.8241-0.9759).

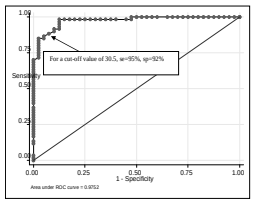
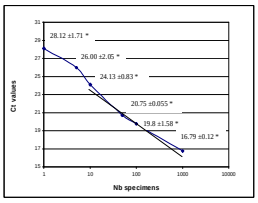


Figure 3: Results of real-time PCR ROC analysis when the morphological determination is used as the gold standard method

Quantification of C. imicola ss specimens



Linear regression of the number of *C. imicola ss* specimens with the Ct values obtained from the real-time PCR method. The standard curve showed a regression coefficient r=0.98, mean squared error with the equation: y=-1.5663 ln(x) +27.21 (without the values of the mean for 1 specimen and with 5 specimens). The average values (SEM) and SD from three independent qPCR reactions are shown *.

Since the samples caught for BT vector surveillance are made of *Culicoides* genus insects and of many other insect families, the analytical sensitivity of the real-time PCR (*e.g.* the minimal number of *C imicola* detectable) was evaluated with the aim not only to detect at least one single specimen of *C. imicola ss* but also to quantify the number of *C. imicola ss* within a pool of other insects The assays repeated in triplicates are highly reproducible with SD values very low (for 10 specimens of *C. imicola ss*, mean of Ct value: 24.13, SD: 0.83; for 50 specimens, mean of Ct value: 20.75, SD: 0.055; for 100 specimens, mean of Ct value: 19.8, SD: 1.58; for 1000 specimens, mean of Ct value: 16.79, SD value: 0.117

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 Ct values in samples containing 1 specimen or samples containing 5 specimens of *C. imicola ss* was not possible. A corresponding Ct value of 30.5 was chosen and therefore gave a relatively high sensitivity of 95% and a specificity of 92%.
Larvae identification by this molecular tool has already been successfully performed (data not shown) and will help for ecology based research. Large scale studies should therefore be undertaken using real-time quantitative PCR to identify breeding sites in the farming environment which are implicated in the spread of the vectors and the disease.

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REFERENCES

Baldet et al., 2004. Vet. Ital. 40 (3), 226-231
Baylis and Mellor 2001. Vet. Rec. 149, 659.
Cêtre-Sossah et al., 2004. Vet Res. 35, 325-37
Delécolle et al., 1985PhD dissertation, Université Louis Pasteur de Strasbourg I n° 293 : 238 pp
Meiswinkel et al., 2004. Proc. Third International Sympos., Taormina, 26-29 October 2003. Vet. Ital. 40 151-159
Mellor, 199. Curr. Top. Microbiol. Immunol. 162, 143-161
Simon et al., 1994. Annals of the Entomological society of America 87, 651-701

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