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ARTICLE

Challenges for the Characterization of Genetically Modified Animals by the qPCR Technique in the Era of Genomic Editing

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ABSTRACT

Characterization of genetically modified organisms through determination of zygosity and transgene integration concerning both copy number and genome site is important for breeding a transgenic line and the use of these organisms in the purpose for which it was obtained. Southern blot, fluorescence *in situ* hybridization or mating are demanding and time-consuming techniques traditionally used in the characterization of transgenic organisms and, with the exception of mating, give ambiguous results. With the emergence of the real-time quantitative PCR technology, different applications have been described for the analysis of transgenic organisms by determination of several parameters to transgenic analysis. However, the accuracy in quantitation by this method can be influenced in all steps of analysis. This review focuses on the aspects that influence pre-analytical steps (DNA extraction and DNA quantification methods), quantification strategies and data analysis in quantification of copy number and zygosity in transgenic animals.

1. Introduction

Technologies for adding exogenous genes to animals have made remarkable progress in recent years and now show promising results in a range of strategies, such as large-scale production of proteins of therapeutic interest [1], production of experimental models for the study of human and animal diseases, zootechnical improvements, regulation of gene expression studies [2]. Since the production of the first transgenic mouse in 1981 [3], significant progress has been made in methods of introducing the gene for transgene in animals. The first transgenic

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approaches were unpredictable, unrepeatable [4] and, invariably, resulting in the insertion of exogenous DNA into the host genome in several locations and in several copies. In some cases, this led to disruption of the gene's function, ectopic and overexpression or underexpression of the exogenous gene [5]. Conventionally, the characterization of these transgenic animals in terms of copy number, integration site and zygosity, was performed using techniques such as Southern blot, fluorescent *in situ* hybridization (FISH) and mating. However, these methods are technically demanding and time consuming. In addition, except for mating, they give mixed results [6]. On the other hand, real-time quantitative PCR (qPCR) has proven to be a reliable, fast and accurate method for determining zygosity [7] and copy number [8] for transgenic animals.

However, with the evolution of genetic engineering, new methods of producing transgenic animals, called Genetic Editing methods such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) have been widely used worldwide for genetic modification of animals [5,9,10,11]. Several of these techniques can introduce single nucleotide changes without integrating foreign DNA and, thus, generate organisms with desired phenotypes. Consequently, these organisms can be indistinguishable from their natural counterparts, since the modifications can resemble entirely random mutations, regardless of whether they are spontaneous, chemically induced or by irradiation [11]. This required updating the techniques of qPCR, DNA sequencing and DNA hybridization to characterize genetically molded organisms produced with the new genomic editing tools. This review focuses on the aspects that influence pre-analytical steps (DNA extraction and DNA quantification methods), quantification strategies and data analysis in quantification of copy number and zygosity in transgenic animals.

2. Analysis of Transgene Integration in Animal Genome

The molecular characterization of the main features that made this a transgenic animal is an essential step. This evaluation aims to characterize the integrity of the inserted sequence in the genome of the specimen, the expression pattern of the transgene to identify its site of insertion (when the transformation is performed by conventional methods), as well as the number of insertions of the expression cassette [7]. Molecular characterization of the transgene copy number and the zygosity would allow inferring the genome receptor stability after gene transformation and the transmission rate of the transgene to generate a F1 (Figure 1).

2.1 Copy Number

Transgene copy number, defined as the number of exogenous DNA insert(s) in the genome is a key issue for transgenic studies, since it is directly relevant to the effectiveness of transgenic event and data interpretation [11]. Theoretically, a single intact copy may be sufficient to produce the recombinant protein. However, the expression level may be correlated with transgenic gene dosage; a higher copy number may result in increased expression [12]. Nevertheless, this observation is not true for all transgenic events, since an exceptionally high copy number may, in fact, result in low expression [13,14]. A high copy number may not only affect the expression level but also the genetic stability of the transgenic locus [15], due to failure in the recombination process during meiosis. According to Mahon et al. [16], the insertion of a large number of transgenes in tandem can result in intrachromosomal recombination, deletion, breakage or translocation of the transgenic.

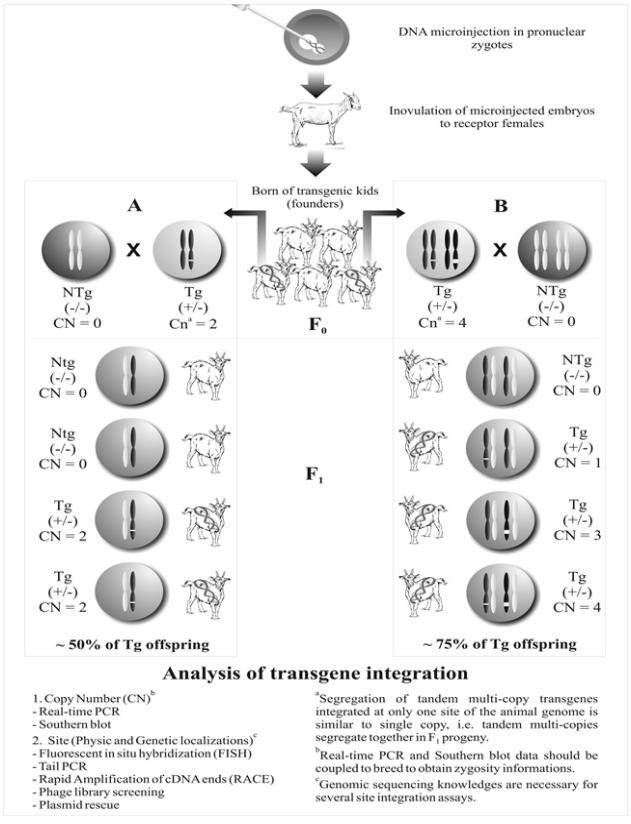

Figure 1. Schematic representation of transgenic animal production by pronuclear microinjection, segregation of the transgene in F1 lineage and methods to analyze transgenic integration.

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After obtaining and characterizing the founding animal, selective breeding allows the establishment of a stable transgenic line. Normally, when the founder animal has more than one transgene integration site, segregation of the transgenes and the creation of independent strains are recommended [7]. However, not always the presence of an intact transgene into the host genome ensures their expression. This is because the expression of a transgene is influenced by its location in genomic DNA, e.g., its position in relation to transcriptional control elements, heterochromatin regions of chromosomes non-transcribed, and other silenced regions [17]. This demonstrates the importance of new genomic editing tools in which site-specific modifications are achieved by targeted cleavage of DNA and homologous recombination using ZFN, which are chimeric molecules, composed of a nuclease domain and specifically designed DNA-recognition domains.

The conventional method for transgene copy number determination is Southern blot hybridization. The usage of a restriction enzyme with only one restriction site in the transgene cassette should be chosen to digest the genomic DNA. Thus, the digested nucleic acid will be used for Southern blot hybridization with transgene specific probes. If Southern blot hybridization renders only one band, the transgene copy number should be one [18-20]. However, Southern blot hybridization requires a relatively large amount of DNA, labor-intensive and time-consuming [20]. Additionally, Southern blot analysis may not be accurate enough to determine copy numbers greater than two [9-11].

The polymerase chain reaction (PCR) is one of the most sensitive methods for detecting the integrated gene in the transgenic animal genome, and thus it can reduce the amount of DNA required for analysis [9,10]. Quantitative PCR has been successfully used to determine the copy number of genes into the genome of many species [8]. In addition, to improve the accuracy of real-time PCR for this application, the most used methods are the external standard curve-based method and the ΔC_T method involving an internal reference gene (Table 1). Ballester et al. [8] described a rapid and accurate qPCR-based system to determine transgene copy number in transgenic animals. The authors used the $2^{-\Delta\Delta CT}$ methods to analyze several mouse lines carrying a goat β-lactoglobulin transgene without the requirement of a control sample previously determined by Southern blot analysis. Instead of a murine DNA, the calibrator was a goat genomic DNA, which was used to amplify both β-lactoglobulin (target) and glucagon (reference) genes. Chandler et al. [21] also used the $2^{-\Delta\Delta CT}$ methods to estimate bacterial artificial chromosomes (BAC) transgene copy number in mice embryos and lines. They observed accuracy and reproducibility in copy number quantification in several of independent transgenic lines and showed that increased BAC transgene copy number is correlated with increased BAC transgene expression. To determine the correlation of transgene expression with copy number, Kong et al. [22] examined the green fluorescent protein (GFP) copy number in ears of newborn and mature transgenic pigs. Interestingly, a decline in copy number was found by both absolute quantitative real-time PCR and Southern blot analysis. In addition, the authors observed a significant correlation between GFP expression levels and copy number in transgenic fibroblast cells. The authors hypothesized that the decline of transgene expression may be due to the loss of copies.

2.2 Zygosity

The zygosity of transgenic animals describes the similarity or dissimilarity of the transgene insertion of homologous chromosomes in a specific allelic position. The term is used to describe homozygous event of double insertion of the transgene in the same position of allelic chromosomes, whereas hemizygote describes the insertion position in a single specific allele. Based on the Mendelian inheritance, the transgene transmission to F1 is considered 100% when the animal is homozygous and 50% when hemizygote. However, animals in hemizygotes with multiple insertion position different allelic can produce the rate of 75% transmission of the transgene (Figure 1). This information is always required for effective breeding and colony maintenance. In addition, heterozygous or homozygous status for the transgene has been shown to correlate with gene expression levels and rates transmission of transgene [7]. When integration takes place at a single location in the genome, in the cell embryo, without compromising the genes involved in gametogenesis that can induce transmission rate distortion, half of the F1 offspring will be transgenic [23]. Normally, the interaction occurs at several different locations in the genome, which may be on the same chromosome or on different chromosomes [23]. When two or more interactions occur on the same chromosome, the rate of segregation of the transgenes or the frequency of recombination during meiotics will be determined by the distance between the integration sites [23].

Results from the zygosity analysis of transgenic animals using qPCR are listed in Table 1. Comparative analysis of zygosity between qPCR and Southern blot analysis in 45 transgenic rats for the human decay-accelerating factor showed ambiguous results when Southern

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blot technique was applied. However, this same analysis using qPCR permitted the clear identification of all transgenic animals as homozygous or heterozygous. Mating of homozygous and heterozygous animals, defined by qPCR, could show transgene transmission to the offspring as expected by Mendelian laws [7]. Shitara et al. [24] developed the system to determine the zygosity using only two experimental processes: estimation of the concentration of DNA and SYBR Green PCR analysis. With this method, the authors successfully discriminated homozygous, heterozygous and non-transgenic animals. In this same work, to confirm the accuracy of zygosity determination by this method, blastocysts obtained from superovulated female mice, which had been mated to male mice of the transgenic strains by *in vitro* fertilization and the green fluorescent protein fluorescence (EGFP) was then visualized under an inverted fluorescence microscope. All embryos (70/70) derived from the F1 male, determined to be homozygous by real-time quantitative PCR, showed EGFP. In embryos derived also from the F1 male, which were determined to be heterozygous, about 55% of these embryos (49/89) showed fluorescence. As expected, these results were perfectly consistent when the used method was qPCR.

3. Real Time Quantitative PCR (qPCR)

The qPCR features such as use of low amounts of template DNA and high specificity due to the high temperature annealing of the primers, compared to low hybridization specificity of the probes in the Southern blot, made this technique a powerful tool in the characterization of transgenic animals. [25]. The high sensitivity is conferred by the exponential nature of the PCR reactions, which enable specific sequences to be detected in samples even if only a few copies are present. The procedure for copy number and zygosity analysis in transgenic animals using qPCR technique can be divided into three steps: pre-qPCR procedures (such as DNA extraction and quantification), qPCR amplifications and post-qPCR procedures (mathematical and statistical data analysis) [26]. Concerning qPCR amplifications, an important issue is the choice of the quantification strategy (with acceptable specificity and sensitivity), which should determine the accuracy of the measurement. The following topics describe these steps

Table 1. Technical details in DNA extraction, quantification, and real-time PCR for copy number and zygosity analysis of transgenic animals.

	Copy number			Zygosity				
	Joshi *et al.* [45]	**Ballester** *et al.* [8]	**Chandler** *et al.* [21]	**Kong** *et al.* [22]	**Haurogné** *et al.* [49]	**Shitara** *et al.* [24]	**Tesson** *et al.* [9]	**Ji** *et al.* [48]
Species	Mus musculus	Mus musculus	Mus musculus	Sus domesticus	Mus musculus	Mus musculus	Mus musculus	Danio rerio
Tissue for DNAg extraction	liver and lung	liver	tail	Fibroblast cells	tail	tail	tail	Tail
DNAg extraction method	P.C.	P.C.	P.C.	Universal Genomic DNA Extraction Kit Ver.3.0	phenol–chloroform protocol	phenol–chloroform protocol	phenol–chloroform protocol	DNeasy Tissue Kit (Qiagen)
Quantification method	UV spectrophotometry at 260 nm	N.E.	UV spectrophotometry at 260 nm	--	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm
Reference gene	β-actin	Glucagon	*Jun* gene	TFRC	Mouse gap junction channel protein alpha 5	--	Rat hypoxanthine phosphoribosyltransferase (rHPRT)	Gene bank AC087105
qPCR chemistry	SYBR Green	TaqMan	TaqMan	SYBR Green	GeneAmp 5700 SYBR Green	SDS 7900 CYBR Green	SDS7700 TaqMan	SDS7700 TaqMan
Normalization	DNA quantification	Endogenous control	DNA quantification	DNA quantification	Endogenous control	Endogenous control	Endogenous control	Endogenous control
Quantification	Absolute	$2^{-\Delta\Delta Ct}$	Absolute	Absolute	$2^{-\Delta\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta\Delta Ct}$	Relative standard curve
Calibrator	N.A.	Mus musculus	N.A.	N.A.	Animal of known zygosity	Animal of known zygosity	Animal of known zygosity	Animal of known zygosity
Quantification method of calibrator	N.A.	Southern blot	N.A	N.A	--	FISH	Southern blot	FISH

N. A. = Not applicable. P.C.= Protocol based on phenol–chloroform method. P. DNAg extraction kit based on spin technology (TaKaRa).

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and the effects in final qPCR accuracy.

3.1 Pre-qPCR Procedures – DNA Extraction

The aim of a nucleic acid extraction method is to isolate DNA of suitable integrity, purity and of sufficient quantity for diagnostic applications by qPCR [27]. Obtaining DNA of high quality is paramount for ensuring confidence in all subsequent steps in the process of generating analytical measurements. However, the quality of the template DNA obtained can vary according to the extraction method used, thereby influencing the accuracy of the quantification.

The basic phenol/chloroform extraction buffer consists of 100 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 200 mM NaCl, 0.2% sodium dodecyl sulfate (SDS) and 0.1 mg/mL proteinase K (molecular biology grade). The phenol/chloroform/isoamyl alcohol (25:24:1) mix is used to remove proteins and polysaccharides and ammonium acetate and ethanol to DNA precipitation [7]. For real-time PCR use, RNA is removed from the nucleic acid preparation with enzymes, as RNase A and RNase T1. However, the prior work of our group demonstrated that this DNA extraction method compromises the efficiency of qPCR [28]. According to published reports [29, 30], phenol/chloroform extracted DNA needs further purification to be used for real-time PCR. The purification of phenol/chloroform extracted DNA with a Genomic Tip 20 column (Qiagen) resulted in a linear calibration curve and produced the expected values [30]. Nevertheless, some studies have succeeded to perform the copy number quantifications and zygosity analysis using this method without purification provided for amplification by real-time PCR [9,10]. Additionally, Sakurai et al. [31] succeeded in the analysis of zygosity in transgenic animals using crude extract obtained from incubation of samples from different tissues of transgenic mice. These controversial results can be attributed to variation in the tissue type used for gDNA extraction.

Two main factors that compromise PCR amplification are: i) the quality of template DNA in the reaction and ii) the presence of a series of inhibitors (Table 2). The presence of inhibitors in the PCR reaction compromises not only the efficiency of the reaction, but also the reproducibility of the PCR, thus contributing to inaccurate qPCR results. The inhibitory mode of action of some of these compounds may be linked with precipitation and denaturation of DNA or the ability of the polymerase enzyme to bind to magnesium ions [32]. Animal tissue or reagents used in the DNA extraction stage, inhibitors generally modify the kinetics of the PCR reaction, chelating Mg^{2+} (a cofactor of DNA polymerases) and / or by binding to template DNA or DNA polymerase [33,34,35]. To overcome this limitation, commercial kits DNA binding to silica-based matrices, followed by elution, can be used to remove inhibitors and organic solvents, such as chloroform. According to Burkhart et al. [34] and our experiments [28], gDNA obtained by silica matrix-based methods are more efficient for amplification by qPCR. An inhibition test using either internal controls or evaluation of the linearity of the calibration curves should be performed to determine the suitability of the extracted DNA for real-time PCR amplification [28, 30].

Table 2. Examples of PCR inhibitors reported and methods to minimize inhibition.

Inhibitors	Description and inhibitory concentration	Methods to minimize inhibition
EDTA	≥ 0.5 mM 1 mM [31]	Reduce the concentration of EDTA to 0.1 mM in the TE buffer or simply use Tris-HCl (10 mM) to bring DNA in solution. DNA can also be brought in pure water (but the DNA cannot be stored for long-term use)
Ethanol	>1% (v/v) [53]	Dry pellet and resuspend
Isopropanol		Dry pellet and resuspend [26]
Protein	1% casein hydrolysate in PCR mixture caused inhibition [31]	Use SDS or guanidinium buffers, proteinase K
	Proteinase K [33]	Non proteinase K based genomic DNA isolation method [33]
Detergents	SDS [53]	Wash with 70% ethanol
Sodium acetate	≥5 mM [53]	Wash with 70% ethanol
Sodium chloride	≥25 mM [54]	Wash with 70% ethanol or use silica-based purification [26]

3.2 Pre-qPCR Procedures – DNA Quantification

Prior to qPCR, stock DNA extracts are commonly quantified and diluted so that all reference and test samples contain identical amounts of this nucleic acid. An accurate determination of DNA concentration in a sample is a critical component for analysis of copy number and zygosity by qPCR. In general, DNA quantification prior to qPCR increases confidence in negative PCR results, where insufficient target DNA could otherwise be interpreted as a false-negative. Two principal methods used for DNA quantification are UV spectrophotometry (absorbance 260 nm – A260) and fluorometry (Table 2). However, the spectrophotometric methods of quantifying DNA do not discriminate intact DNA, RNA and free nucleotides, [36] on the other hand, fluorescent dyes, are highly specific for double-stranded DNA. [37] According to Shokere et al. [36] A260 and fluorescent–dye methods of quantifying intact genomic DNA provide relatively concordant DNA quantification values. However, the quantification values differ significantly for an identical DNA extract that has been

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degraded with its non-degraded counterpart. This study revealed that A260 values overestimate by an average of 20.3% (± 6.1) and fluorescent–dye methods underestimate by an average of 145.8% (± 6.0). The DNA concentration of PCR-amplifiable intact DNA extracts. Furthermore, when fluorescent–dye methods of DNA quantification were compared with A260 methods, an average percent difference of 10.1% (± 6.3) was reported for intact genomic DNA, but a much more significant percent difference of 152% (± 10.3) was reported in degraded genomic DNA. [36]

3.3 qPCR Amplifications – Quantitative Strategies

Target nucleic acids can be quantified using either absolute or relative quantification. The absolute quantification determines the absolute amount of target, whereas relative quantification determines the ratio between the gene target amounts in two samples (an unknown and another previous quantified, named calibrator) [38]. For relative strategies of quantification, in general, is necessary to amplify an endogenous reference gene (usually an appropriate housekeeping gene) in the two samples of DNA, to normalize the data [39]. All qPCR methods demand to plot standard curves for mathematical validation of gene amplifications. A standard curve is generated using a dilution series of at least five different concentrations of the DNA template. [40] The most important parameters calculated with the plots are: 1) linearity (Pearson correlation coefficient, R^2) – must be greater than 0.96 and it is also important to make PCR reactions with DNA amounts that are within the linear range of amplification. [28] 2) Efficiency (E) – must be close or equal to 1.0 (ideal values are between 1.1 and 0.9). [41] When all these requirements are fulfilled, both methods can be successfully used to estimate the number of copies or zygosity in transgenic animal. The work of our group demonstrated that high error rate ranging from 11-177% in absolute quantification, when these requirements are not met [28].

3.3.1 Absolute Quantification

Absolute quantification (Figure 2) can be achieved by a relation of the C_T measurement to a standard curve that can be obtained by diluting a standard DNA sample (as a plasmid) with the transgene sequence for which the exact DNA concentration and molecular weight is known [42]. The C_T values can thereby be related to a distinct number of plasmids and with the knowledge of the molecular weight of the haploid animal genome, the number of molecules represented by a certain amount of animal DNA can be estimated [28, 43]. For this, it is necessary to construct a standard curve using serial dilutions of at least five different concentrations. The amount of unknown target should fall within the range tested. Amplification of the standard dilution series and the target sequence is carried out in separate wells. The C_T values of the standard samples are determined. Then, the C_T value of the unknown sample is compared with the standard curve to determine the amount of target in the unknown sample. A given number of animal DNA molecules yield the same C_T value as the same number of plasmids, if all molecules contain one copy of the transgene (i.e., if the animal is homozygous). [28] For heterozygous animal with only half of the molecules containing the expression cassette, the C_T value will count for half of the number of plasmids. [28] This method was validated by Schmidt and Parrott [43]. The advantages of this method are that large amounts of standard that can be produced, its identity can be verified by sequencing and DNA can easily be quantified by spectrophotometry or fluorometry. Plasmid standards should be linearized since the amplification efficiency of a linearized plasmid often differs from that supercoiled conformation and more closely simulates the amplification efficiency of genomic DNA. Additionally, due to variations in inhibitor levels of qPCR between tissues is recommended also the use of plasmids mixed with genomic DNA samples of non-transgenic animals to delineate the curves to simulate possible interferences of each tissue [44].

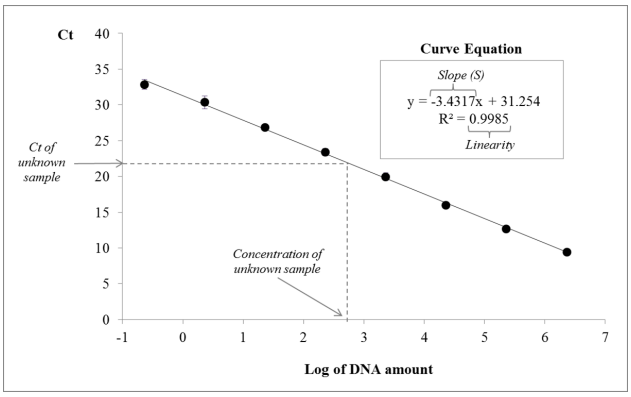

Figure 2. Typical standard curve and principles of absolute quantification strategy. The C_T values were plotted *versus* DNA amounts used for qPCR amplifications. The slope of the tendency curve achieved by linear regression is used to determine the efficiency of qPCR. The Pearson correlation coefficient (R^2) is the linearity and should be close to the unit (or 100%). Determination of a sample of interest (unknown sample) is performed by extrapolating the C_T value on the standard curve.

The gDNA can also be used to construct the standard curve for absolute quantification. However, in this situation, analysts need to certify the presence of only one

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copy per haploid genome of the target DNA and the exclusion of closely related pseudogenes and / or sequences from amplification. The main advantage of this approach to quantification is that there is no need to use a calibrator (a sample for which the copy number is exactly known, typically from a Southern blot). However, the accuracy of this strategy is directly associated with the precision in DNA quantification. As discussed above, currently, the main DNA quantification methods are spectrometry (A 260 nm) and fluorometry, which can, respectively, overestimate and underestimate the real amounts of DNA. Consequently, these matters will reflect in the C_T values, compromising the result [35]. This imprecision in the result can be even more pronounced when the plasmids are used to construct the standard curve, due to the small mass of this deoxyribonucleic acid (small errors in the quantification reflect in large variations in the C_T). However, several groups have used this method successfully for the characterization of transgenic animals (Table 2). Using, this strategy to estimate the number of copies of transgenic mice for hG-CSF, we observed an accuracy of 100% when DNA was quantified with fluorometer and qPCR all requirements have been met [28].

3.3.2 Relative Quantification

The comparative C_T is the most used relative quantification method for several purposes, including transgene analysis [10, 44]. However, the validation of comparative C_T for transgene copy quantification requires a previous comparison between standard curves plotted for the transgene (target) and the reference gene (Figure 3). Thus, the efficiency ($E = 10^{(-1/Slope)}$) of both transgene and reference gene amplifications must be highly similar. A simple way to determine what relative quantification method can be applied is to plot ΔC_T values (calculated as the difference between target and reference gene C_Ts) versus log of DNA amounts. The comparative C_T method can be used if the slope of this plot is between -0.1 and 01. However, if the slope is out of this range, the indicated method should be the relative quantification by standard curves. Another limitation of using this strategy to copy number quantification and zygosity analysis is referred to the accuracy of the results of the quantification calibrator. The main justification for the use of real-time PCR in the characterization of transgenic animals is the increased reliability of results obtained in relation to conventional methods, as Southern blot, and FISH [8,9,10]. However, copy number and zygosity analysis in calibrator animals are usually performed by conventional methods. In this sense, any errors that may have occurred in the characterization of this animal will be impressed in real-time PCR quantification of target animals.

3.3.3 Relative Quantification by Standard Curves

The characterization of transgenic animals using these methods requires the use of a reference sample, with the number of copies or the known zygosity, and an endogenous control gene [46]. The amount of the target is determined from the standard curve of the transgene and an endogenous control. Normalization is performed by dividing the equivalent dilution of the transgene by the equivalent dilution of the endogenous control [47]. Standard dilution equivalents without a unit require a sample to serve as a calibrator. A good calibrator for quantifying copy number is a homozygous animal with one copy of the transgene. Samples with half the normalized dilution equivalent used as the calibrator are heterozygous; samples with the same normalized dilution equivalent are homozygous for one copy, and so on. The quantification procedure differs depending on whether the target and the endogenous reference gene are amplified with comparable or different efficiencies. This strategy has been used mainly to determine the zygosity of transgenic animals [25, 47, 48].

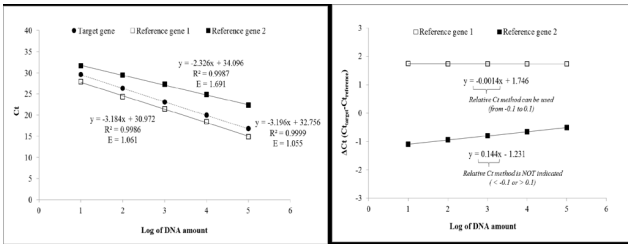

Figure 3. Determination of real-time PCR efficiencies from the slopes of the calibration curve. To compare the amplification efficiencies of the 2 target sequences, the C_T values of reference gene 1 are subtracted from the C_T values of target gene. The difference in C_T values is then plotted against the logarithm of the template amount. If the slope of the resulting straight line is < 0.1, amplification efficiencies are comparable.

3.3.4 Relative Quantification by Comparative C_T Method

While requiring an endogenous control and a calibrator, differs from the relative standard method by relying on equal PCR efficiencies with the transgene and the endogenous control genes. The preparation of standard curves is only required to determine the amplification efficiencies of the transgene and endogenous control genes in an initial experiment. In all subsequent experiments, no standard curve is required for quantification of the target sequence. According to Livak and Schmittgen [40], if all amplicons amplify with the same efficiency, the difference

ΔC_T between the C_T for the transgene (C_Tt) and the C_T for the endogenous control (C_Te) is constant, independent of the amount of chromosomal DNA ($\Delta C_T = C_T t - C_T e$). As for quantification with relative standards, a calibrator is a homozygous one-copy animal. Thus, all samples with the same ΔC_T as the calibrator contain one copy of the transgene. More generally, the ratio of the initial amount of transgene in the sample (Xs) to the initial amount of transgene in the calibrator (Xcal) can be calculated as follows ($Xs/Xcal = (1+E)^{-\Delta\Delta CT}$), where: $\Delta\Delta Ct = \Delta C_T s - \Delta C_T$-cal. Whereas for copy number calculation $\Delta\Delta C_T s$ will be zero (one-copy animals) or negative (multi-copy animals), zygosity analysis should yield $\Delta\Delta C_T s$ of zero (homozygous) or one (heterozygous). As long as the efficiencies for transgene and endogenous control are the same, calculations with E<1 are also possible. The $2^{\Delta\Delta CT}$ method is simple to apply because DNA concentrations do not have to be measured. Its utility has been demonstrated for animal copy number determination [8] and zygosity analysis in animals [7].

3.5 Post-qPCR Procedures – Mathematical and Statistical Considerations

Due to the high variation in CT values, it has been proposed that the limit for determining the copy number of the transgene by the qPCR technique is two-fold differences. According to Bubner and Baldwin [43], when the standard deviation of the CT values for all samples and amplicons is greater than 0.3, the interpretation of the transgene copy numbers will be compromised, dividing the inability to detect differences twice. Mason et al. [51] reported that only about 70% of qPCR-based transgene copy determination results could be verified by Southern blot analysis. In addition, another aspect that affects the determination of the copy number by qPCR is the lack of complete statistical analysis and adequate models, capable of testing the hypotheses [12]. Normally, hypothesis tests were not invoked for the transgene copy numbers, in addition, a predetermined P value and the confidence levels of the estimate were not specified. These aspects, especially the confidence levels, are important for determining the number of copies of the transgene, since it defines the precision and sensitivity of the assay [52]. Due to the limitations of statistical procedures, the results of the analysis are often ambiguous and without clear confidence intervals. The confidence intervals help to establish the reliable interval for the estimate of ΔΔCT, while the value of P determines the level of significance [53]. In the statistics program, all P values are derived from the null hypothesis test that ΔΔCT is equal to 0. Therefore, a small P value indicates that ΔΔCT is significantly different from 0, which demonstrates a significant effect. Despite this, some studies have shown accuracy in the characterization of transgenics. Haurogné et al. [49] reported success in estimating the copy number for CT differences close to 1, when they used low concentrations of genomic DNA. In summary, small fluctuations in the initial conditions of a PCR assay led to a large fluctuation in the amount of the product, which is expressed in CT (or equivalent) values. Thus, apparently small standard deviations of the CT values (between 0.3 and 1) are amplified in the analysis because a difference of CT of one represents a difference of twice the initial value. In addition to the variability in transgene measurements, the variability in the measurement of endogenous control must also be considered [50].

4. Conclusions

In summary, this review allows us to conclude that real-time PCR is a powerful tool for the characterization of transgenic animals, especially for copy number determination and zygosity analysis. This quantitative technique has the potential to become a widespread tool in animal transformation research, because it helps to characterize the lines, to infer or to explain transgene expression levels and to drive the reproductive managements for livestock establishment. However, the choice of the quantitative PCR method must be accompanied by appropriate validations, ensuring that the measurements are correct and adjusted to the experimental conditions (genes, primers, templates, temperatures). Finally, specific transgenic animal lines that are considered for research or commercial release probably will require the confirmation of both copy number and zygosity data by independent methods, such as Southern blot and mating, respectively. Hence, real-time PCR is a tool that complements rather than replacing traditional procedures.

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ARTICLE

Antibacterial and Antioxidant Activity of *Rhodomyrtus Tomentosa* and *Cinnamomum Zeylanicum* Crude Extracts

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ABSTRACT

The aim of this study was to investigate the extraction method for *R. tomentosa* and *C. zeylanicum* leaves and the evaluation of antibacterial and antioxidant activities of crude extracts. The results of the study showed that the active ingredients of crude extracts were clearly separated by Thin-layer chromatography and the presence of rhodomyrtone in *R. tomentosa* crude extract and cinnamaldehyde in *C. zeylanicum* crude extract. *R. tomentosa* crude extract was antibacterial activity against *Staphylococcus aureus* with 13.1 mm of inhibition zone, but is not effective against *Salmonella* Typhimurium. *C. zeylanicum* leaf extract did not show antibacterial activity on both *S. aureus* and *S*. Typhimurium. At a dilution of 1/2 of the *R. tomentosa* crude extract can completely inhibit *S. aureus* growth. This study also indicated the presence of antioxidant compounds such as flavonoids, tannins, phenols and terpenoids in *C. zeylanicum* and *R. tomentosa* crude extracts. The results showed that *R. tomentosa* and *C. zeylanicum* crude extracts should be used as a biotherapy alternative to antibiotic therapy. However, further study would be needed to investigate the antibacterial activity of crude extracts *in vivo*.

1. Introduction

The use of herbs and medicinal plants as the first medicines. In recent years, multiple drug resistance in human and animal pathogenic microorganisms has developed due to indiscriminate use and commercial antibacterial drugs commonly used in treatment. This situation encouraged scientists for searching new alternative substances from various sources like medicinal plants which are the good sources and novel antimicrobial chemotherapeuticagents. Likewise, antioxidants play an important role in protecting cellular damage by reactive oxygen species. The medical plant is the most important targets to search for natural antioxidants from the point of view of safety.

R. tomentosa and *C. zeylanicum* have long been used in Oriental medicine. In human medicine, *R. tomentosa* leaves have long been used to treat diarrhea, relieve pain, stop bleeding wounds, or some diseases of the urinary tract [7, 8]. Meanwhile, *C. zeylanicum* leaves are effective for flatulence, indigestion, nausea, abdominal pain, diarrhea, gastrointestinal spasm and gastrointestinal disturbances [6]. *R. tomentosa* leaf extract and *C. zeylanicum* leaf extract were reported to have good antibacterial, anti-inflammatory, anti-fungal and antioxidant activity [5].

The present study was, therefore, aimed at evaluating the antimicrobial and antioxidant activity of *R. tomentosa*

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and *C. zeylanicum* leaf extracts against some pathogenic microbes. The results of research should be applied to the prevention and treatment for animal diseases.

2. Materials and Methods

2.1 Herbal Plants Preparation and Extraction

R. tomentosa and *C. zeylanicum* leaves were collected from Phu Yen province – South Central Coast Vietnam. The leaves were then washed under running tap water and shade dried at 60 °C for 48 hours, then grinded into powder.

A total of 50g of powder was dissolved with 200 ml of ethanol solvent and then centrifuged (5000 rpm / 15 minutes). The supernatant was then evaporated at 60 °C for 30 minutes [8], and screened for antimicrobial and antioxidant activity.

2.2 Analysis of Active Ingredients of Crude Extracts

Crude extracts of *R. tomentosa* and *C. zeylanicum* were loaded on a thin plate of aluminum backed silica gel 60 F254 (Merck, Germany) on the semi-automatic thin plate chromatography system (Camag, Switzerland), the blasted plate was dried naturally at room temperature, then placed in a 20 x 10 cm twin trough chamber (Camag, Switzerland) containing the developing solvent which is toluene: ethyl acetate (93: 7) [16].

The interpretation of separated active compounds was observed by UV chamber (Camag, Switzerland) with 256 nm wavelength [1].

2.3 Identification of Pharmacological Active Ingredient (Rhodomyrtone for *R. tomentosa* and Cinnamaldehyde for *C. zeylanicum*)

Rhodomyrtone was determined by comparing movement coefficient (Rf) of chromatographic streak corresponding to *R. tomentosa* leaf extract sample with Rf of positive control rhodomyrtone (rh) (SMB00114, purity ≥ 95%, Sigma, USA).

Cinnamaldehyde was determined based on the comparison of the Rf of the chromatographic streak corresponding to the *C. zeylanicum* extract with the Rf of cinnamaldehyde according to the study research has been published [16].

2.4 Antibacterial Activity

The antimicrobial activities were done by using bacteria strain like *Salmonella* Typhimurium (ST) and *Staphylococcus aureus* (SA). The antimicrobial activity was determined by disc diffusion method. The Mueller Hinton agar plates were inoculated with a bacterial suspension (adjusted to 1-3 x 10^8 CFU /ml). 20 µl of extracts were loaded onto sterile paper disks and placed on the culture plates. 20 µl of amoxicillin + clavulanic acid (Nam Khoa Company) was used as control. Then the plates were kept for incubation at 37°C for 24 hours. At the end of incubation, the diameter of inhibition zones around the discs was measured.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Standard bacteria suspension *S. aureus* at 600 nm (OD = 600) (equivalent to 10^5 CFU/ ml).

The crude extract was diluted into 3 concentration levels: undiluted (1), diluted 1/2 and 1/4. Determine the minimum inhibitory concentration of *R. tomentosa* extract using the 96-well microplate described by Sultanbawa et al. [15].

2.6 Determination of the Antioxidant Activity

The presence of antioxidant compounds such as flavonoids, tannins, phenols and terpenoids in *C. zeylanicum* and *R. tomentosa* crude extracts were determinated by chemical reactions [6].

3. Results and Discussion

3.1 Analysis of Active Ingredients of Crude Extracts

The results of the separation on the thin plates of crude extract of *R. tomentosa* and *C. zeylanicum* leaves (Figure 1) showed that the number of chromatographic streaks separated from the *R. tomentosa* leaf extract sample was 8 streaks and the *C. zeylanicum* leaves were 12 streaks. Thus, the number of chromatographic streaks separated from *C. zeylanicum* leaf extract is higher than *R. tomentosa*. Furthermore, the streaks appearing in the chromatogram of the sample was *C. zeylanicum* darker and clearer than that of *R. tomentosa* leaf extract. This shows that the leaf extract of *C. zeylanicum* contains more active ingredients than the *R. tomentosa*.

3.2 Determination of the Presence of Rhodormyrtone and Cinnamaldehyde

Research results from Figure 2 show the ability to detect the presence of rhodormyrtone and cinnamaldehyde in extracts of *R. tomentosa* and *C. zeylanicum* by TLC. Rhodomyrtone in *R. tomentosa* leaf extract, used experimentally in many different studies, has been shown to reduce the invasion and adhesion of *S. aureus* in the subcutaneous tissue of bovine udders, which is an important

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property in the treatment of mastitis in dairy cows in clinical and subclinical form [7,8]. Meanwhile, the active ingredient cinnamaldehyde in *C. zeylanicum* leaves has also been shown to be resistant to many foodborne pathogens [6].

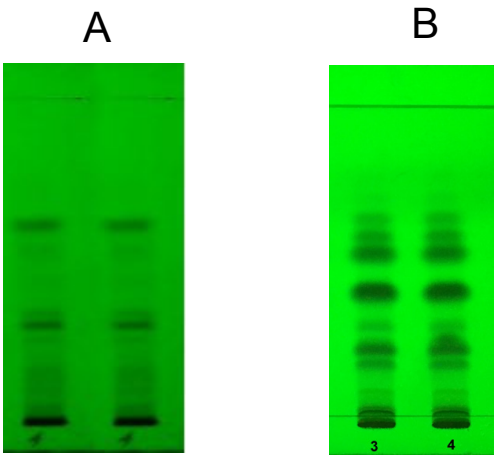

Figure 1. Results of separation of active ingredients in *R. tomentosa* and *C. zeylanicum* leaf extracts observed with UV (λ= 254 nm). A: *R. tomentosa*; B: *C. zeylanicum*

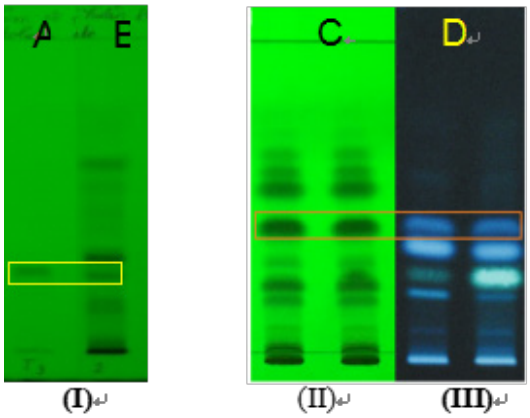

Figure 2. Results of separation of active ingredients with pharmacodynamic activity from *R. tomentosa* leaf (I), *C. zeylanicum* leaf (II) observed with UV (λ= 254 nm) and *C. zeylanicum* leaves according to Wagner et al. [16] (III). A: standard rhodomyrtone; B: *R. tomentosa* leaf extract; C: *C. zeylanicum* leaf extract; D: *C. zeylanicum* leaf extract [16].

3.3 Determination of Antibacterial Ability from *R. tomentosa* and *C. zeylanicum* Leaf Extracts

Table 1. Results of antibacterial ring diameter (mm) of *R. tomentosa* and *C. zeylanicum* leaf extracts for *S. aureus* and S. Typhimurium

Extract (20 µl)	*Staphylococcus aureus*	*Salmonella* Typhimurium
R. tomentosa	13.1 ± 0.8	0
C. zeylanicum	0	0
AMC	33	13

For *R. tomentosa* leaf extract, presence of rhodomyrtone was confirmed through a chromatographic streak on a thin plate with standard rhodomyrtone. For *C. zeylanicum* leaf extracts, the determination of cinnamaldehyde was based on the movement coefficient Rf (= 0.40) and chromatograms of Wagner et al. [16].

From the results of separation of active ingredients and the presence of two active ingredients with pharmacological activity: rhodormyrtone and cinnamaldehyde *R. tomentosa* and *C. zeylanicum* leaf extracts showed the applicability of TLC in the detection of valuable active ingredients in pharmacology.

Testing the antibacterial susceptibility assay by disk diffusion test on agar showed that the inhibition zone of *R. tomentosa* leaf extract against *S. aureus* strain was average at 13.1 ± 0.8 mm compared with the control inhibition zone of amoxicillin + clavulanic acid (AMC) at 33 mm (Table 1). According to Mordmuang et al. [4], paper plate containing 2.5 mg crude extract of *R. tomentosa* leaf extract showed an inhibition diameter of 8.7-15.5 mm for *S. aureus* in the study of antibacterial activity.

However, the antimicrobial of *R. tomentosa* leaf extract has not been observed when tested with *Salmonella* Typhimurium. In a study by Kusuma [4], *R. tomentosa* leaf extract for the inhibition zone of *Salmonella* typhi is about 15 mm in diameter. However, there have not been many more clear tests on the antibacterial activity of *R. tomentosa* leaf extract to *Salmonella typhi*murium.

While *R. tomentosa* leaf extract was effective against *S. aureus*, in contrast, *C. zeylanicum* leaf extract did not show the ability to inhibit both *Staphylococcus aureus* and *Salmonella typhi*murium. *C. zeylanicum* leaf extract had no effect on *Salmonella typhi*, but had good effects on *Escherichia coli, Bacillus subtilis, Candida albicans, Klebsiella pneumoniae* [2].

3.4 Minimum Inhibitory Concentration (MIC) of *R. tomentosa* Leaf Extract for *S. aureus*

Table 2. Minimum inhibitory concentration of *R. tomentosa* leaf extract against *S. aureus*

Active elements	Minimum inhibitory concentration (MIC)		
	Time (hour)		
	18	24	48
R.tomentosa extract (dilution)	½	½	½
Amoxicillin (µg /ml)	12.5	12.5	12.5
AMC (µg /ml)	6.25	6.25	6.25
Ceftiofur (µg /ml)	6.25	6.25	6.25

Control: concentration of the antibiotic is 200 μg /ml

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Based on the antibacterial activity of *R. tomentosa* leaf extract against *Staphylococcus aureus*, the study continued to determine the minimum inhibitory concentration (MIC) of *R. tomentosa* leaf extract against *S. aureus* (Table 2). *R. tomentosa* crude extract at 1 and at 1/2 dilution gave the ability to completely inhibit *S. Aureus* according to the method of Sultanbawa et al. [15]. The use of crude extracts has advantages such as an easy access to *R. tomentosa* leaves, fast extraction process, and the extract can be used immediately after extraction. According to research by Saising et al. [11], the minimum inhibitory concentration of *R. tomentosa* leaf extract ranged from 512 µg /ml for *S. Aureus* isolated from the field. As for bacteria strain *S. Aureus* ATCC 25923, the value is 32 µg / ml [11]. Meanwhile, according to Mordmuang et al. [8], the MIC value of *R. tomentosa* leaf extract for *S. Aureus* isolated from mastitis cows in Canada was 16 **µ**g /ml.

MIC results of ceftiofur and amoxicillin + clavulanic acid (AMC) for *S. Aureus* were lower than amoxicillin (6.25 µg /ml versus 12.5 µg /ml) after 3 times of investigation. This shows that *S. Aureus* were more sensitive to ceftiofur and AMC than amoxicillin. *R. tomentosa* leaf extract at 1/2 dilution gave antibacterial abilities equivalent to antibiotics at a concentration of 12.5 µg /ml. The latest research by Mordmuang et al. [7, 8] was conducted to test the injection of *R. tomentosa* leaf extract on the mammary glands of the rat population with the *S. Aureus*. Results of the study showed that *R. tomentosa* leaf extract with a concentration of 300 µg /ml was injected directly into the mammary gland to help reduce the concentration of bacteria. *S. Aureus* is an important pathogenic bacteria in veterinary medicine and particularly the main cause of mastitis in cows - the most costly economic disease in dairy industry in the world.

The results of this study showed that the crude extract of *R. tomentosa* has good antibacterial activity against *S. Aureus*. Therefore, using *R. tomentosa* leaf extract can be an alternative to antibiotics in the treatment of diseases caused by *S. Aureus*. To do this, it is necessary to have follow-up studies in vivo to test the effectiveness of *R. tomentosa* leaf extract.

3.5 Determination of the Antioxidant Activity

The result shows the presence of flavaniods, tannins, terpenoids and phenol in both *R. tomentosa* and *C. zeylanicum* leaf extracts. The research of Hasibuan et al. [3], also showed similar results with the very high content of terpenoids and phenols in the extract of *R. tomentosa*.

According to study results of Mazimba et al. [6], in the extracts of *C. zeylanicum* leaves are rich in flavonoids, terpenoids, tannins and phenols. The presence of these active ingredients explains the medicinal properties of *R. tomentosa* and *C. zeylanicum* such as: antibacterial, anti-inflammatory, anti-allergic, diabetes treatment, pain relief as well as central nervous system support.

4. Conclusions

In conclusion, the active ingredients in *R. tomentosa* and *C. zeylanicum* leaf extracts good by TLC. The antimicrobial activity against *S. Aureus* of rhodomyrtone in *R. tomentosa* leaf extract and the presence of flavaniods, tannins, terpenoids and phenol in both *R. tomentosa* and *C. zeylanicum* leaf extracts should be applied in therapy fields such as bovine mastitis, dermatis, respiratory diseases.

The future study should investigate the antimicrobial activity against *S. Aureus* of rhodomyrtone in *R. tomentosa* leaf extract *in vivo* and the side effects of the active ingredients in *R. tomentosa* and *C. zeylanicum* leaf extracts.

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ARTICLE

Determination and Prevalence of Ticks in Cattle in Konya Province of Turkey

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ABSTRACT

Ticks are common in the world. Diseases caused by ticks and fleas bring significant economic losses to the livestock industry. With the pathogens they carry, Blood-fed ticks infect humans and domestic animals. This study was conducted between January 01 and August 30, 2018, in the Konya province of Turkey, to determine the prevalence and species of ticks in cattle. 272 pieces of cattle were examined in terms of tick infestations. These cattle were selected from herds of 16 different cattle breeders in 5 different regions of Konya. Ticks were collected by the simple random sampling method. Tick infestation was detected in 70 (25.7%) pieces of cattle that were examined during the study.

Tick infestation was followed in 68 (29.3%) pieces female cattle and 2 (5%) pieces male cattle. During the study conducted, the following results had been determined; according to age, 12 (14.5%) of ticks were juvenile, 58 (30.7%) of them were adults, according to the body condition, 26 (23.4%) of them were good, 35 (26.1%) of them were average and 9 (33.3%) of them were week.

It is found that cattle in the study area were infested in the tick species *Rhipicephalus (Boophilus) annulatus* 65/272 (23.9%) and *R. bursa* 5/272 (%1.8). 332 female and 304 male total of 636 ticks were collected from the cattle. Genders were determined under a stereomicroscope. The high tick infestation shows that fight against tick is a hard process, and planning is a must to reduce the tick burden in cattle. Besides, this study will enable us to make suggestions to the relevant sectors in terms of parasitic struggle in eliminating the health and economic problems caused by ticks in the Konya province by determining the prevalence and species.

1. Introduction

Cattle breeding is very important for the Turkish economy. Animal products are raw materials of food, leather, and textile industries. Konya province is one of Turkey's most important livestock hubs. In Turkey, there are 18 million cattle. In terms of meat and milk production and cattle population, Konya covers 5% of the country's requirements [1].

Ticks cause serious health problems and economic losses in cattle. Ticks can cause restlessness, low productivity, growth retardation, inability to gain weight, irritation, toxic-allergic reactions, anemia, and even death. The number

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of parasites, age of the host, feeding status, and climatic conditions determine the degree of parasite damage to the host [2-5]. It has been reported that ticks are the most common vectors for human and animal diseases worldwide after mosquitoes [6,7]. Along with the direct effects of ticks on animal production and productivity, they transmit more than 200 bacteria, viruses, protozoans, and rickettsia-borne diseases to humans and domestic animals [8].

It is stated that out of 896 tick species diagnosed worldwide, 702 of them are Ixodidae (hard ticks), 193 of them are Argasidae (soft ticks) and 1 is from the Nuttalliellidae family [9,10]. In Turkey, the Ixodidae family is common except *Anocenter* and *Amblyomma*. Common lineages are some species of *Hyalomma, Rhipicephalus, Haemaphysalis, Ixodes*, and *Dermacentor* [10]. In every development period, ticks suck blood [5,6], and they cause damage to the skin of hosts [11]. Without sucking blood, larvae can survive for two months, nymphs for one year, and adult ticks for three years. Depending on the species, ticks live between 6 months and 3 years. During the day, they hide in nooks and crannies, and the plaster crevices and cracks on the wall. Females lay 25-100 eggs at a time, 200-15,000 in total [6,8,11,12,13,14,15].

Single-hosted ticks (for example, *Rhipicephalus spp*.) stays on the same host during all development stages. Double-hosted ticks (for example, *Hyalomma spp*. and *R. bursa*) stays on one host during the larval and nymph stages then stay on another host during the adult stage. Triple-hosted ticks (for example, *D. marginatus*, *Ixodes ricinus* and *Haemaphysalis punctata*) stays on different hosts during three development stages [8].

Ticks can easily detect the host by tracking chemical substances such as ammonia emitted by the host, host vibrations, body temperature, and the carbon dioxide they exhale [8].

Ticks are common in our country and in different countries in the world. Ticks cause significant economic losses due to loss of yield, death, and treatment costs [16,17]. By determining the prevalence and types of ticks in the Konya province, this study will be beneficial to the relevant sectors and the public in terms of fighting against diseases and reducing economic losses.

2. Materials and Methods

2.1 Study Area

The study had been conducted in Turkey's largest city of Konya and its districts, within a total area of 40.838 sq km (Figure 1). Konya is located between 36°41 'and 39°16' north latitudes, and between longitudes of 31°14'and 34°26' east. The elevation of Konya is 1016 meters above sea level. Konya is one of the important cities with a 926.217 cattle population [1].

Figure 1. Physical map of Konya (study area marked with red dots).

2.2 Study Period and Cattle

The study was conducted between January 1, 2018, and August 30, 2018. 272 cattle which were selected from the herds of 16 different cattle breeders and raised via traditional methods, were selected via the sampling method.

2.3 Determination of Sample Size

The required sample size was calculated according to the formula below [18].

$$n = \frac{(1.96)^2 P_{exp}(1 - P_{exp})}{(d)^2}$$

2.4 Sampling Methods

Simple random sampling method was used. The samples were inspected by randomly selected 272 cattle on farms that were told by the breeders that ticks were present without any ectoparasite control. Ticks found in cattle were collected. The age, sex and physical appearance of tick-infected cattle were noted during physical examination [19]. Cattle age was determined by checking the tooth structure [20].

2.5 Collection and Protection of Ticks

Cotton with 70% alcohol was pressed on ticks that were

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detected by macroscopic examination of cattle to prevent mouth organelles to remain on the skin of the host. The ticks were collected by pincer on the skin and placed in vacuum tubes containing 70% alcohol. The species, gender, age, sample collection time, the general name of the parasite, the name of the cattle owner, and the sampling location were written on the collection tubes and labeled [4,6]. In our study, ticks were generally collected from the ear, mouth, neck, chest, back, inner and outer parts of the leg, lower chest, and tail.

2.6 Identification of Ticks

The tick samples were brought to Selçuk University Faculty of Veterinary Medicine, Department of Parasitology Laboratory. Ticks were made transparent in lactophenol and then fixed on a slide with Canada Balsam. With the help of identification tools in the relevant literature, ticks were examined under stereo microscopy and identified at the species level according to their morphological characteristics [2,3,6,12].

2.7 Data and Statistical Analysis

Data recorded in field studies evaluated by SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) statistical package program. n and % are used to introduce the variables. Categorical data are relationships between the determinants of ectoparasite prevalence and prevalence percentage. Categorical data were analyzed by Fisher's Exact Test and Chi-Square test. For the significance level of the tests, $p < 0.05$ was accepted [21].

3. Results

272 cattle identified in the study areas were examined for tick presence. Tick prevalence was 25.74% (70/272) in cattle (Table 1). In terms of species two tick species, *R. (B.) annulatus* 23.90% (65/272) and *R. bursa* 1.84% (5/272) were identified (Table 1). 636 adult ticks (332 females and 304 males), were collected from the cattle (Table 2).

Table 1. Tick distribution in the Konya province

External parasites	Cattle n=272	
	Number	%
Rhipicephalus (Boophilus) annulatus	65/172	23.9
R. bursa	5/272	1.8
Total	70/272	25.7
n: number of examined animals		

Table 2. Distribution of tick species collected in cattle in Konya province

External parasites	Gender			Total
	Male n=304	Female n=332	Number of Ticks	%
R. annulatus	286 94%	311 93.7%	597	93.9
R. bursa	18 6%	21 6.3%	39	6.1
Total	304	332	636	100
n: number of collected ticks				

In terms of the gender of the host, *R. annulatus* was detected more in females (27.6%) than males (2.5%) (Table 3). In terms of age, *R. annulatus* was detected more than twice in adult cattle (29.1%) than juvenile (12%) (Table 3). In terms of the host body condition, it was determined as weak (33.3%), average (26.1%), and good (23.4%), respectively (Table 3).

R.bursa tick species were rarely detected in both male (2.5%) and female (1.7%) cattle. Tick infestation rates by age and body condition are demonstrated in the table (Table 3). In our study, the significance level of the distribution of ticks in cattle according to age, sex, and body condition was $p < 0.05$.

Out of 597 detected *R. annulatus* ticks 286 of them are male and 311 of them are female. Out of 36 detected *R. bursa* ticks 18 of them are male and 21 of them are female. No significant difference was found between the female and male numbers of the collected ticks (Table 2)

Table 3. Percentage distribution of tick species in cattle by sex, age and body condition, number of cases

External parasites	Gender		χ^2 and P-value	Age		χ^2 and P-value	**Body Status**			χ^2 and P-value
	Male n=40	Female n=232		Juvenile n=83	Senior n=189		Good n=111	Average n=134	Week n=27	
R. annulatus	1 2.5%	64 27.6%	5.701 and 0.017	10 12.1%	55 29.1%	1.981 and 0.159	25 22.5%	31 23.1%	9 33.3%	2.088 and 0.358
R. bursa	1 2.5%	4 1.7%		2 2.4%	3 1.6%		1 1%	4 3%	0	
Total	2 5%	68 29.3%		12 14.5%	58 30.7%		26 23.4%	35 26.1%	9 33.3%	
n: number of examined animals										

It was found that ticks cause redness, bleeding spots, and abscessed lesions on the skins of some cattle.

4. Discussion

40-50% of the daily protein requirement of humans should be met from animal-based foods to have a balanced diet and adequate nutrition [1,22]. In many countries, animal production is one of the major driving forces of economic development. Besides, animal husbandry contributes to social and economic development and the formation of a healthy society, such as supporting a balanced and adequate diet, an incentive to exports, supplying raw materials to factories, reducing unemployment in production and service sectors [22]. Therefore, detection of ticks and effective fight against them will reduce economic losses and health problems.

There are many studies to detect ticks in cattle around the world and Turkey. This study finds outs that, tick infestation was common (25.7%) in cattle. Different studies around the world reported (16-89.4%) general tick infestation in cattle [23-29].

In Turkey, different researchers reported (9.5-48.9%) general tick infestation in cattle [18,19,31-36]. Our study shows that *R. annulatus* (23.9%) was the most common species. The rate of *R. bursa* is (1.8%).

In the studies conducted in different parts of the world, different types of ticks have been identified than those identified in our study. For example; *Hyalomma detritum* (84.3%) [37] is the most common species in Tunisia, *R. bursa* and *H. m. marginatum* are the most common species in the Macedonian region of Greece. *Boophilus annulatus, H. detritum scupense, R. turanicus, R. sanguineus, I.gibbosus, H. anatolicum excavatum, I. ricinus, D. marginatus, Hae. inermis, Hae. punctata*, and *Hae. sulcata* are other identified species [38]. *A. variegatum, H. m. marginatum, H. rufipes, H. truncatum, H. nitidum, R. annulatus, R. muhsamae, R. senegalensis, R. sulcatus, R. turanicus, B. annulatus*, and *B. geigyi* are found species in the Republic of Guinea [39]. In Japan, notified tick species are *Hae. longicornis, A. testudinarium, B. microplus, Hae. flava, Hae. kitaokai, I. ovatus, I. persulcatus* [40]. *Hyalomma anatolicum anatolicum* and *H. a. marginatum* tick species are found in the Dohuk province of Iraq [41].

Tick infestation in the Peshawar region of Pakistan is (20.4%) in cattle and detected species are *Boophilus* (43.4%), Hyalomma (36.7%), *Rhipicephalus* (16.9%), and *Amblyomma* (3.1%) [23]. This is similar to the general prevalence rate of ticks in our study (25.7%). Tick infestation in cattle in Iran is (57%), and detected ticks are *Rhipicephalus, Haemaphysalis* and *Dermacentor*, *Boophilus*, and *Ixodes* lineages [24]. *B. microplus* (40.7%) was found in cattle in the Uttaranchal state of India [42]. *A. variegatu* (38.2%), *H. rufipes* (18.4%), *H. truncatum* (15.3%), *R. appendiculatus* (11.9%), *R. evertsi evertsi* (6.3%), *B. decoloratus* (5.6%), *R. praetextatus* (2.2%), *B. annulatus* (1.5%), *A. lepidum* (0.4%) and *R. sanguineus* (0.2%) were identified in cattle in South Sudan [43]. Study conducted in Gazipur Bhawal region of Bangladesh shows that (64.07%) of cattle hosts ticks. Reported ticks are *B. microplus* (45.63%), *R. sanguineus* (36.89%), *Haematopinus euysternus* (17.96%), *Hemaphysalis bispinosa* (16.50%) have been reported [25]. It was determined that tick infestation in studies conducted in Iran, India, Sudan, and Bangladesh was much higher than our study. *B. decoloratus* (45%), *A. coherence* (24.4%), *R. evertsi* (15.6%), and *A. variegatum* (15%) were collected from cattle in the Assosa region of Ethiopia [44]. Identified and reported ticks (89.4%) in the West Amhara region of Ethiopia in cattle are *A. variegatum* (49.2%), *B. decoloratus* (21.2%), *H. marginatum* (9.8%), *Hya. truncatum* (6.2%), *R. evertsi* (6.6%) and *R. pulchellus* (5.3%) [26]. Tick infestation in cattle in the Bench Maji region of southwestern Ethiopia is (16%), and identified types are *B. decoloratus* (8%), *Amblyoma variegatum* (4.7%), *A. coherens* (4.2%), *Haematopinus euysternus* (3.8%) [27]. In the Bishoftub region of Ethiopia, the infestation is (40.1%) in cattle, and reported tick species are *Amblyomma* (67.6%) and *Boophilus* (32%) [28]. It is reported that cattle in the Gondor region of Ethiopia are infested with *Boophilus* (6.81%), *Amblyomma* (2.92%), *Hyalomma* (5.84%), and *Rhipicephalus* (1.94%) [45]. Except for one, four separate studies conducted in Ethiopia, indicate that the general tick prevalence is higher than the rate in our study. Detected ticks in cattle in the Karakorum, Pakistan are (77.9%), and as species, *Hyalomma anatolicum* and *R. microplus* were identified [29]. According to a study in Punjab province of Pakistan detected ticks in cattle are (42.4%) and most of these are *H. anatolicum* (29%) [47].

It is understood that the rate of tick infestation detected in two different studies conducted in Pakistan is two to three times higher than the rate indicated in our study. A study conducted in the Gazipur Bhawal region of Bangladesh, indicate that the prevalence was higher in the seniors, and females were more infested with ectoparasites than males, and the parasite infestation rate in cattle in the free rearing system was higher than in other cattle breeding system. All of these are similar to our study [25].

Turkey also researched different regions to detect ticks in cattle and the commonly reported species are *Haemaphysalis, Hyalomma, Boophilus, Dermacentor, Rhipicephalus* and *Argas* [47].

Tick infestation in the study conducted in Van City in

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Turkey is (48.88%) and *H. excavatum*, *H. anatolicuın*, and *Dermacentor* spp. are identified species [48]. According to our study, the prevalence is very high and the species are different. In the study conducted in Kayseri, tick infestation was (17%) and *R. turanicus*, *R. sanguineus*, *R. annulatus*, *R. bursa*, *H. anatolicum anatolicium*, *H. a. excavatum*, *H. detritum*, *Hae. sulcata*, *Hae. parva*, *D. marginatus*, and *O. lahorensis* are identified species [49]. The determined prevalence rate is close in our study, only two species *R. annulatus* and *R. bursa* are found similar. *Hae. parva* (33.8%), *B. annulatus* (21.1%), *H. marginatum* (19.7%), *Hae. concinna* (15.5%), *R. bursa* (7%) and *D. marginatus* (2.8%) were identified in the Sivas-Zara region in Turkey in cattle [50]. In our study, the two identified species are similar. Species of *Hyalomma*, *Rhipicephalus*, and *Haemaphysalis* in cattle (14-57%) have been reported in the study conducted in the Elazig province in Turkey [30]. Prevalence was similar in our study, and only *Rhipicephalus* was found as lineage. Tick infestation in cattle (29.9%) in the study in Malatya in Turkey and the surrounding area [31] is similar to our study. In the Burdur province, ticks in cattle are (21.8%), and reported species are *R. turanicus*, *R. annulatus*, *H. marginatum*, *Hae. parva*, *D. marginatus* [32]. The infestation was similar in our study, *R. annulatus* was similar to the species. A study conducted in Ankara City in Turkey indicates ticks are (19.16%) and identified species are *Hae. parva*, *Hae. Punctate* and *Hae. sulcata* [33]. The infestation rate is close to our study, and different tick species are identified. In Aydın, Izmir and Manisa Cities of the western Aegean region of Turkey, reported species are *H. marginatum* (37.39%), *H. excavatum* (18.89%), *H. detritum* (13.68%), *H. anatolicum* (0.86%) and H. rufipes (% 0.07) [51]. These are completely different from the tick species identified in our study area. In the study conducted in Van and Ercis of Turkey, the infestation is (37.5%) in cattle and 12 tick species are identified [34]. The infestation rate and variety of species are very high compared to our study. In the Kütahya province of Turkey, ticks are found in (9.5%) of the cattle and the identified species is *R. annulatus*, *D. marginatus*, *Hae. parva*, *Hae. punctat*, *Hae. sulcata*, *H. marginatum*, I. hexoganus, I. ricinus, *R. bursa*, *R. sanguineus* and *R. turanicus* [35]. Prevalence is low but the number of species identified is very high compared to our study. In the Afyon City of Turkey, the infestation is (18.12%) in cattle, and reported species are *R. bursa*, *R. sanguineus*, *R. turanicus*, *H. marginatum*, *H. detritum*, *H. excavatum*, *D. marginatus*, D. niveus and *Hae. sulcata* [36]. The prevalence is similar in our study, as specie with only similar identification is *R. bursa*. Another study reported that *R. bursa* is widespread in the Mediterranean and the Black Sea regions of Turkey [52].

A study conducted in the year 1988 in the Konya province of Turkey reported *R. bursa*, H. a. anatolicum, *H. a. excavatum*, *H. detritum*, and *R. turanicus* species [53]. Compared to our study, *R. bursa* is the only similar spicy among five species belonging to the reported two lineages. In another study conducted in the year 2006 in the Konya province of Turkey, identified species are *R. bursa*, *R. turanicus*, *R. sanguineus*, H. a.anatolicum, *H. a. excavatum*, *H. m. marginatum*, I. ricinus, *D. marginatus*, *O. lahorensis* and *Hae. parva* [54]. One can say that identified species is very high compared to our study and the only concordance is seen for *R. bursa*.

Turkey is located in the subtropical climate zone. Since there is a variety between regions, diseases transmitted by ticks are seen in all regions.

Rhipicephalus bursa, *R. turanicus*, *R. sanguineus* and *R. (B) annulatus* is a significant number in Turkey. *R. bursa* completes the development process mostly in sheep, goats, cattle and horses. *R. (B) annulatus* is mostly seen in cattle, domestic ruminants such as sheep and goats.

As it is seen in other parts of Turkey, both *R. bursa* and *R. (B) annulatus* was determined in cattle in Konya. *Rhipicephalus bursa* species is a vector for *B. ovis*, *B. bigemina*, *B. bovis*, *B. caballi* and *T. ovis*, while *R. (B) annulatus* is a vector for Babesia bigemina, *B. bovis* and Anaplasma marginale. *R. sanguineus* is a vector of *B. canis*, *B. gibsoni*, *Ehrlichia canis*, *Rickettsia rickettsi* and *Hepatozoon canis*. *Rhipicephalus* sanguineus species is a vector of *B. canis*, *B. gibsoni*, *Ehrlichia canis*, *Rickettsia rickettsi* and *Hepatozoon canis*.

I. ricinus it is seen in the coastal regions of Turkey. It carries disease factors such as *Babesia bigemina*, *B. divergens*, *B. microti*, *Anaplasma phagocitophylum*, *Borrelia burgdorferi*, Tick borne encephalitis virus, louping-ill virus. *Ixodes ricinus* that are important vectors of terms in Turkey could not be identified in this study because it is not beachfront Konya region.

Hyalomma spp. has in every climate zone in Turkey significantly affect human and animal health. *H. anatolicum*, *H. detritors H. excavatum*, *H. marginatum* and H. aegyptium is widely seen in Turkey, *Hyalomma spp*. has not been observed in cattle in this study. *H. marginatum* corresponds to Crimean-Congo Hemorrhagic Fever virus, *H. anatolicum* is the vector of *Theileria annulata* and *T. equi (B. equi)*.

Haemaphysalis parva, *Hae. punctat*a, *Hae. sulcata* and Hae. inermis have been identified In Turkey, While *Haemaphysalis* species are more common in humid and temperate regions, Konya region has not been seen due to hot and dry summers and cold and rainy winters. *Haemaphysalis* species are vectors for *Anaplasma centrale*,

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Anaplasma marginale, *B. bigemina* and *Theileria buffeli/orientalis*.

Dermacentor marginatus, *D. niveus* and *D. reticulatus* have seen in Turkey. These species are vectors for *Babesia caballi*, *T. equi (B. equi)* and *B. canis*. These species are not found in cattle in Konya region [7,8].

It is understood from studies that tick infestation is still at high levels in many parts of the world. It is considered that the prevalence of tick infestation is high in Turkey and the world because of drug resistance and inadequate parasite control. Climatic conditions in the research areas, precipitation, the feeding and sheltering conditions of the animals, and tick collection time interval could be effective in facing different tick species and variability of their prevalence. Besides, conducting the study before or after the parasite control may differ the results [15].

Although there is success about spicy diversity in external parasite control in the study areas *R. annulatus* is still widespread (23.9%). This important fact indicates that there are still things to do in tick control. The reason why some tick species are not observed in our research area could be the result of the transition to closed system (barn) animal husbandry and excessive ectoparasite control. However, this may bring into mind that tick species may also be seen in unexamined herds in the same region.

5. Conclusions

This research determines that tick infestation still threatens animals at high rates. The main reasons for these are; ticks hide in places where acaricides cannot reach, host selectivity of ticks, their ability to suck blood from every creature, their ability to survive for years even in adverse weather conditions, and the difficulties in their fight and control due to their resistance to acaricides. If even a small number survive after the pesticide application, many new tick infestations may occur thanks to their high reproductive ability. Besides, the fact that wild animals are effective in feeding and spreading ticks aggravates tick control and cause unsuccessful results. Thus, eradication of ticks is not fully possible [7,13].

Ticks attack the host only to feed and leave the host after feeding [4,5]. Therefore, these protection measurements should be taken; new animals entry into the herd should be controlled, the animals should be well fed and cared for, the animals should not be sheltered in closed areas for a long time, and the animals in the shelters should be ranged on sunny days [4,11].

To fight against the ticks and diseases caused by ticks, fighting methods based on species of ticks, their prevalence, hosts they prefer, their effects as vectors, the risk map about which hosts they use as reservoirs should be determined [7]. It is important to prevent ticks from contact with animals in the prevention of zoonotic diseases transmitted by ticks. As a result, it is ensured that diseases transmitted by ticks do not pass to animals. Thus, it is ensured that zoonotic diseases are not transmitted from animals to humans. It is of great importance to provide personal protection in preventing the spread of these diseases.

As a result of the research, when the potentially harmful effects of ticks in terms of health and economy are evaluated, it will shed light on future studies as this is an up-to-date problem in the region.

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Ethics Committee Approval

Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee was approved by decision number 2017/178 dated December 29, 2017.

Authors Contribution

This research is designed and directed by U. Uslu. A. Küçükyağlıoğlu collected ectoparasites from the specified animals. Genre identification and statistical analysis are conducted by U. Uslu and A. Küçükyağlıoğlu. This article is prepared by A. Küçükyağlıoğlu.

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REVIEW

Evidence-based Rapid Review to Approach Diagnostic Test Research. A Veterinary Practitioners Opinion

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ABSTRACT

In animal research systematic reviews and meta-analysis have been playing an important role in improving the quality of evidence that professionals use worldwide. However, it is claimed that it is in its initial stage of development. In veterinary medicine the heterogeneity in the evaluation of variables of exposure and response makes it difficult to gather the data results for a meta-analysis and evidence-based rapid reviews and other types of reviews can accelerate the way how we obtain this information and a problem-solving approach can be developed in the veterinary medicine field.

1. Introduction

Systematic reviews and meta-analysis are the best types of studies to report evidence-based medicine with high quality standards and greater strength in their conclusions compared to other types of studies [4;14]. There are numerous possible interventions in human and veterinary medicine that meta-analysis can resolve regardless of whether the conclusions of the analysis are positive or negative [11]. A meta-analysis carried out by collecting the effect size from similar studies offers the possibility to be updated when new research is published. Besides, gathering effect size can impulse other researchers to report it, rather than relying on the traditional p-value.

In animal research systematic reviews and meta-analysis have been playing an important role improving the quality of evidence that professionals use around the world and there is an updated database (VetSRev) to avoid repeated publications, the authors encourage readers and researchers to consult this database before beginning a new systematic review or meta-analysis (http://webapps.nottingham.ac.uk/refbase/). The objective of systematic reviews and meta-analysis in veterinary practice is to always provide the best possible evidence in any type of intervention for animal health and animal husbandry. However, unlike human medicine, in veterinary medicine these types of scientific advances are not well developed, indeed, it is claimed that it is in its initial stage of development [7].

2. Thinking Outside the Box

Currently, the collection of information can be accomplished in a timely manner in human medicine. However, in veterinary medicine, it is often time consuming and

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arduous to at least obtain a sample size large enough to guarantee quality results [7]. Furthermore, in veterinary medicine there is often not a sufficient number of homogeneous clinical trials, making it difficult to analyze the information to produce reliable results [3].

The characterization of diagnostic tests and its ability to recognize the truly positives, the truly negatives and specifically define disease or health status in an animal, is carried out in studies with different experimental designs and statistical methods, such as reporting the area under curve, the sensitivity and specificity, effect size or setting up the reference intervals where there is a healthy control group and an affected group characterized with the diagnostic test and a gold standard test [12]. However, it is not correct to use a diagnostic test immediately after this kind of characterization despite offering excellent results.

Regardless of encouraging results of a diagnostic test, the studied population does not necessarily behave the same as another in different settings. Therefore, considering the variability of the population, it is advisable to evaluate the same test in different situations, such as in vitro, in vivo, in situations of natural disease and induced disease, but also to establish possible events that may alter the diagnostic method and its results.

To reach a rational conclusion regarding the quality of a diagnostic test, it is important to have results that can be evaluated and compared under different conditions and with a seemingly similar population to define the size of the effect, and which strength of recommendation will be defined in order to recommend it in practice. In veterinary medicine, this situation almost never occurs and there is a gap regarding the rigor that veterinarians have to recommend and to use a diagnostic test in veterinary practice [9].

In some cases, only one cohort with small animal population is used, with different types of studies and sometimes, based on empiricism and weak results. In addition, it is quite frequent that in veterinary medicine, researchers do not take into consideration the effect size and overestimate the importance of the p-value to report statistical significance or difference between study populations.

Considering the above, it is clear that in veterinary medicine the heterogeneity in the evaluation of variables of exposure and response makes it ever more difficult to gather the data results. Indeed, there is an evident gap related to the scarcity of existing research in order to create new, purposeful and conclusive meta-analysis and systematic reviews that can be reproduced [8]. There is a critical need for developing clinical guidelines that outline specific and appropriate diagnostic tests and treatments in order to reach or approach a decisive conclusion. This goal is frustrated by the existence and common usage of variable methodologies within different clinical trials with the same objective.

In recent years, we have chosen to classify the evidence provided by the publications where diagnostic tests are analyzed to recommend them at the practical level. This has increased the quality of veterinary medicine practice and has generated a tendency to find new alternatives, comparable to systematic reviews and meta-analysis and the direction that scientific research is currently looking forward is a path where medical knowledge can be provided with a more agile and faster way to obtain conclusions, but keeping the credibility and reliability of scientific evidence [14,5].

3. Opportunity for Evidence-base Rapid Reviews

The world is constantly changing and new emerging diseases such as SARS-CoV-2, African Swine Fever (ASF) or Leishmaniasis create an opportunity to modify the way we create novel investigations. This emerging and re-emerging diseases need regular updates to generate good quality knowledge in a short period of time. Therefore, evidence-base rapid reviews have grown in popularity since the last decades and they have many algorithms to facilitate the review process and make the research development shorter and more agile [13,6]. However, should be stated that this type of study gives relevant results in a shorter period of time, could have bias or limited accuracy in the results because it simplifies or omits the conventional systematic review process recommended in the PRISMA statement [14].

In general, there are different sorts of evidence-base medicine review process with results characterized as umbrella reviews [1], review summaries, a summary of systematic reviews and also a synthesis of reviews [13]. There are also scoping reviews that have been used for the last decade gaining strength to categorize different types of studies in terms of their impact and scope in different areas, especially diagnostic test. In addition, one of the features of this sort of evidence-base medicine reviews is that is possible to identify and maintain control over the flow of information in any field of knowledge, helping researchers to improve the typology of studies in veterinary medicine [10].

Apparently in veterinary medicine greater control is required in terms of study methodologies and characterization of variables conducting impact and scope reviews such as those previously mentioned. The evidence-based rapid reviews as brief approach should be considered as a premature field in veterinary medicine. Thus, further strengthened and updates are expected because these stud-

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ies do not yet have an essential uniformity due to the lack of guidelines as the PRISMA statement for systematic reviews and meta-analysis. The future is promising for this kind of research and the transmission of information for decision-making will become fast and uniform with the cutting-edge methods of obtaining knowledge [2].

4. Conclusions

Systematic reviews and meta-analysis cannot be replaced in terms of evidence-base medicine, at least for the upcoming years. It's likely that artificial intelligence and machine learning can and will generate new methods to obtain results without bias with greater strength of recommendation and better quality of evidence with minimal human intervention. However, in the meantime systematic reviews and meta-analysis will remain critical to achieve the objectives of medical practice worldwide in order to always have the most accurate and reliable possible evidence to obtain the best results on the target population and evidence-based rapid reviews and other types of reviews can accelerate the way how we obtain the information and a problem-solving approach can be develop in the veterinary medicine field.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the research.

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