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INTRODUCTION

The use of PCR (polymerase chain reaction) is becoming increasingly common in the world of avian diagnostics. Veterinary practitioners are using cost-effective commercially available PCR-based diagnostics for a variety of purposes including sex determination, pathogen identification and pathogen detection. In the future, screening for genetic predisposition to certain diseases using PCR is also likely to become affordable for veterinary use.

With the rapid increase in the use of PCR has come an increasing need for clinicians to understand what it is that PCR does and how to interpret results in a way that is meaningful for the management of cases. An understanding of how inaccurate results may occur and how to avoid and control for them is essential, although as laboratories become more stringent in sample submission, handling and processing procedures the emphasis is falling increasingly on clinicians to correctly interpret the results of PCR.

PCR: AN OVERVIEW

PCR is little more than the selective amplification of DNA or RNA. The technique was developed in the early 1980's in response to the need for larger amounts of DNA to enable downstream applications such as sequencing and electrophoresis (Saiki et al., 1988). The development of practically applicable PCR is popularly accorded to the Californian Kary Mullis in 1993, for which he was awarded the Nobel Prize in Chemistry.

In order to amplify DNA or RNA a **template** is required. In the context of veterinary diagnostics this is derived from the sample which was collected and submitted to the laboratory. Prior to PCR itself the DNA or RNA in the sample is extracted and purified for use as the template. Samples, such as faeces, that may contain abundant inhibitors of the PCR process may require specific template preparation techniques to maximise the limits of detection of the assay (Takashima et al., 1996).

In the presence of free nucleotides, buffering agents and the co-enzyme magnesium chloride the enzyme **DNA polymerase** (which is used by cells to replicate) is capable of extending strands of DNA complementary to the template. A small strand of DNA complementary to a specific site in the template must be provided in the reaction, from which the DNA polymerase can start its strand, these small strands (oligonucleotides) being called **primers**. The distance between primers oriented in different directions along the double stranded DNA of the template defines the length of the new DNA strand that the DNA polymerase synthesises during the PCR.

The double stranded nature of most DNA and the inability of primers to anneal (bind) to sites on DNA which is in this state first requires the **denaturation** of the template, whereby at high temperatures (typically 95°C) the two complementary strands of DNA denature (melt) apart to form single strands. Once this occurs the temperature of the reaction is reduced to between 40°C and 72°C so that **annealing** of the primers with their specific sites can occur before the reaction is increased to 72°C which is the optimum temperature for timely synthesis of complementary DNA strands by the DNA polymerase starting at the primers (a process called **extension**). The end result is the original single stranded DNA bound to its newly synthesised complementary strand. In order for the process to continue, and for more strands to be synthesised, denaturation must occur again before annealing and then extension is possible.

One of the early challenges for PCR was that most DNA polymerases become inactive at the high temperatures needed to denature double stranded DNA. A solution was found in the form of DNA polymerase isolated from the bacterium *Thermus aquaticus* (*Taq*), which lives in thermal springs and as such has evolved a heat tolerant form of the enzyme. *Taq* DNA polymerase and variants of it are now used universally to enable many cycles of denaturation, annealing and extension in one PCR for the dramatic exponential amplification of the primer-determined sites of DNA.

It is worth briefly mentioning that this process is similar for RNA (which in the veterinary diagnostic context is most often being amplified for the detection of RNA viruses) except that an initial step called **reverse transcription** is used in which the enzyme reverse transcriptase converts the RNA in the template to DNA, which the DNA polymerase can then copy. Note that the abbreviation RT-PCR is often used for reverse-transcription PCR to detect RNA as well as Real-Time PCR which are two different processes.

The duration of a PCR varies depending on the amount of template present, the nature of the primers, the particular attributes of the DNA polymerase used, and the length of the strand being amplified, but usually is between one-and-a-half and three hours. At the end of the reaction the amplified DNA is appropriately called **product** or **amplicon**. This product is then used for downstream applications such as sequencing or simply gel electrophoresis, in which strands of DNA of different length in the product are separated out and visualised.

In terms of diagnostics, PCR is mainly used to determine the presence, and sometimes the genotype, of particular DNA or RNA (usually belonging to a pathogen). Visualisation of a product of expected length after a PCR in which highly-specific primers for a pathogen, such as *beak and feather disease virus* (Ypelaar et al. 1999), have been used is occasionally sufficient. Often however the identity of an organism or gene sequence cannot be confirmed simply with the presence or absence of product of appropriate length and further steps such as sequencing of the product is needed to make the diagnosis. An alternative is the **nested PCR** which is a two step procedure that relies on using the amplified primary product as a new template for specific intra-amplicon targeting (Takashima et al., 1996).

When multiple gene sequences of interest or different pathogens are to be detected in a single sample type laboratories may use a **multiplex PCR** to amplify all target sequences in the same reaction and thus minimise assay time and consumables. However this often results in a decrease in the limits of detection for each individual target sequence (Messmer et al., 1997; Freick et al., 2008).

PCR: THE RISKS

Numerous risks for error exist in sample collection and handling, in the PCR itself and in the interpretation of results from PCR (Olsen and Speer, 2009). Most of these relate to the highly sensitive nature of this procedure: PCR amplifies DNA, and this amplifies also the effect of contamination, which is an every present concern when using this diagnostic method.

False positives

PCR is often used in the testing of a sample (such as blood, body fluid or tissue) for the presence or absence of DNA or RNA specific to a pathogen. The degree of specificity in the reaction depends greatly on the nature of the primers. Where primers are highly specific to a site only present in the pathogen of interest, the synthesis of an appropriate PCR product is indicative that DNA from that exact pathogen was present in the reaction. Where primers are more general to a group of organisms (this can range from several species to entire phyla) all that can be inferred from the same result is the presence of DNA in the reaction which is derived from some organism from that group. While not truly a 'false' positive, if this organism was not the pathogen being tested for the result can be misleading, and great care is needed in the interpretation of such a result based on the specificity of the primers being used.

One of the greatest procedural risks in PCR is contamination of the template with target DNA. This can come from either unintended contamination of samples with environmental material (principally during sample collection and handling before submission to a laboratory) or by contamination of the template in the laboratory with PCR product from previous reactions (Bonne et al., 2008). Contamination from these sources is a very real concern and great care should be taken to avoid it.

The risk of contamination of samples by environmental DNA depends on the nature of the DNA being tested for. Some pathogens for which there is copious shedding of infectious material (such as BFDV) preclude the use of any material exposed to the environment (such as feathers) for meaningful PCR diagnosis in individuals which are not isolated for a prolonged period. For most PCR protocols the collection of tissues protected from environmental contamination (such as blood) are ideal, so long as these tissues are appropriate for the question being asked (this is discussed further below).

Laboratory contamination is another matter entirely and while many labs that routinely run PCRs will control and monitor for contamination carefully, this is not always the case and there can be great variation in laboratory testing procedures as well as results (Olsen and Speer, 2009). The onus is on clinicians to ensure the results they have been provided are accurate, and they can do so by asking their laboratory to confirm that suitable controls have been used in every PCR. The most likely sources of contamination in the laboratory are cross-sample carryover of DNA during template preparation (demonstrated spectacularly with PBFVDV using standard preparation protocols) and contamination of reactions with product (which itself can act as a template) from previous PCRs. The reality is that most molecular labs are aware of these issues and should use negative controls in every batch from template preparation to product visualisation or quantification. A laboratory should be able to justify the use of controls, or lack thereof, to clients.

False negatives

While rarer than false positives, false negative results in PCR do occur (Khalesi et al., 2005). There are various reasons why a PCR will not produce product from a sample that contained DNA or RNA of

interest. Most importantly however, the clinician needs to consider whether the sample collected was appropriate for the question being asked, and whether an adequate volume of the appropriate tissue was submitted for PCR to be able to detect target DNA or RNA which is at a very low concentration or unevenly distributed in tissues. Occasionally the concentration of target material is so low relative to the DNA content of the host tissue (such as with some haemoparasites at very low abundance) that it is extraordinarily difficult to provide enough pathogen DNA for a PCR without overwhelming the reaction with host DNA.

While DNA is surprisingly robust the same cannot be said for RNA. Where the target for a PCR is an RNA virus for instance, great care must be taken to collect and store samples appropriately (often by freezing immediately or mixing with special buffers that protect the RNA). The reason this occurs is due to the almost ubiquitous presence of RNAses, which degrade strands of RNA. DNAses exist but are less of an issue. For instance, dry swabs stored at room temperature will often be suitable for DNA amplification after prolonged periods. More caution is needed when dealing with wet tissues or material containing live microorganisms. No matter what sample is being collected, it is best to contact the laboratory running a PCR for guidance on appropriate storage and handling.

Occasionally the reason for a negative result is that highly specific primers have been used for a PCR which are unable to bind to DNA from the target organism. This is especially common when looking for suspected pathogens in a new host or where the pathogen may differ genetically from the most common variant. Degenerate primers which are more relaxed in their annealing site requirements are often necessary, although not always successful, in these situations. The amplification of circoviruses in different avian hosts (such as ravens) for example required this approach (Todd et al., 2001; Johne et al., 2006; Stewart et al., 2006), where a standard primer pair used for a known sequence such as BFDV would not amplify anything.

While elegant in its simplicity and wondrous to behold when it works, anyone who has slaved for any serious amount of time with DNA will assure the uninitiated that PCR, as with most molecular procedures, is a dark and mysterious art which will promptly cease working for no apparent reason. This is not a great concern for the patient clinician, as long they have been careful to avoid contamination during sample collection and their laboratory has included positive controls with every batch (right from template preparation to product visualisation or quantification) to ensure that a negative result is sample specific and not because a reaction or template preparation failed. Clinicians should enquire about the inclusion of positive controls where dubious negative results occur.

Interpretation

Problems with false positive or negative PCR results can usually be avoided through appropriate sample collection and handling and through the use of controls in the laboratory, which clinicians should ensure is the case. PCR is typically very accurate and in most cases a greater issue exists with the interpretation of results than with inaccurate results, a responsibility which lies very much with the clinician.

The most important two things to bear in mind when interpreting PCR results are what exactly the result is telling you, and how this relates to the question being asked. The latter should be considered before PCR is used, but this is not always the case.

A PCR result tells you nothing more than whether a fragment of DNA or RNA, defined by the primer pair used, is present or absent in the reaction. In the absence of any error as described above this

can be inferred to be indicative of the presence or absence of that fragment in the collected sample.

First and foremost for avian practitioners, the specificity of the primer pair used must be considered. If the primer pair is known to be ruthlessly species- or even strain-specific, a positive result can usually be inferred to indicate the presence of DNA or RNA from the specific organism in the collected sample. A negative result is harder to interpret, particularly in unusual host species or with little known pathogens where cryptic genetic diversity may prevent successful primer binding, even between quite closely related organisms. With primers that are suitable for a wide group of organisms more confidence may be placed in a negative result, however all that can be inferred from a positive is that DNA or RNA from any one of the organisms which the primer pair are suitable for is present in the collected sample. The use of the common trichomonad primer pair TFR1 and TFR2 developed by Felleisen in the 1990's is, for instance, often used erroneously to infer the presence of specific organisms such as *Trichomonas gallinae* when in fact all that can be inferred from a positive result is that DNA from any one of hundreds if not thousands of known and unknown parabasalid parasites is present in the collected sample (Felleisen, 1997). Where any doubt exists about the identity of a positive PCR result, the product from the reaction must be sequenced for meaningful interpretation.

It is significant that PCR can only inform as to the presence or absence of target DNA or RNA but not live organisms in a sample. The presence of such genetic material must be parsimonious with infection for the result to be meaningful (Lazizi and Pillot, 1993). For instance, finding of fragments of trichomonad DNA in the oropharynx of a bird of prey which has recently consumed a squab is in itself insignificant. Without further evidence of the clinical relevance of trichomonad DNA at this site (such as the presence of motile flagellated protozoans on wet prep or abscessation in the oral cavity) this finding means nothing more than just that: fragments of trichomonad DNA, whether from live or dead organisms, are present in the oropharynx of the bird of prey. Likewise the finding of DNA specific to viruses of arthropods in the intestinal wall of an insectivorous bird is not in any way indicative that the bird was infected by such a virus, as such DNA could quite parsimoniously be found in phagocytic cells found in such tissue. Where DNA or RNA from a pathogen is not detected, the clinician needs to consider whether the sample collected was likely to contain genetic material from the suspected pathogen in the tested individual. The message here is that the clinician has to understand pathogen and host and to think very carefully about the circumstances under which pathogen DNA or RNA may have ended up in, or avoided, the sampled host tissue.

CONCLUSION

There can be little doubt that PCR will continue to be an important aspect of avian veterinary diagnostics. The technique is highly sensitive and is likely to remain one of the most powerful and reliable diagnostic methods available, provided that clinicians and laboratories prevent and control for inaccuracy and consider what it is that a PCR result is actually telling them.

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