Diagnostic porcine polymerase chain reaction assay: The future is here

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Summary

This report discusses the relative importance of polymerase chain reaction to veterinary diagnostics. A brief description of the assay procedure is included.

To devise proper treatment and prevention programs, one must be able to accurately detect and identify porcine pathogens. One should judge the relative merit of the many diagnostic tools available to accomplish this on the basis of the "three Ss"—speed, sensitivity, and specificity. Polymerase chain reaction (PCR) is a new assay that promises improvement in all three Ss[2,3] over the most commonly used diagnostic methods.

In terms of the three Ss, serology and isolation have some inherent limitations:

- they can be slow—it can take days to months to obtain results;
- the sensitivity of these methods is relatively low;
- they can only be used with a limited sample range (tissue, feces, or blood);
- they require that the samples be fresh; and
- they cannot detect some organisms, such as swine hepatitis E and papilloma virus.[1]

Polymerase chain reaction is the wave of the future in diagnostics, because it increases sensitivity (it can detect as few as 10 organisms in a sample), specificity, and speed (it can be completed within several hours). Any type of sample can be used with PCR, which identifies pathogen (virus, bacteria, or parasite) and host genes. Thus, the PCR assay allows us to detect pathogenic organisms that may not have been detectable using other methods.

Some critics of PCR consider the technique too sensitive, because PCR detects the pathogen's genetic material whether the pathogen is dead or alive. Polymerase chain reaction can result in false positives from vaccine strains, contaminates, normal flora, or nonviable organisms.

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This diagnostic note has been refereed.

This is the first in a series of three articles describing diagnostic techniques. It is available online at **http://www.aasp.org/shap.html**.

The increased specificity of PCR, however, can also be exploited to differentiate vaccine strains from field strains. This specificity is achieved with primers—short fragments of synthetic DNA that are 18–25 bases long. These primers are designed to match a unique region of the target DNA. If successfully designed, the assay can differentiate a field strain from a vaccine strain. In studies with porcine reproductive and respiratory syndrome virus (PRRSV), vaccine-specific primers detected the vaccine strain months after the initial vaccination.[4]

Sampling

Many different sample types can be used for the PCR assay—serum, whole blood, urine, semen, and tissues. The optimal sample will depend on the organism of interest and the specific PCR assay implemented. To reduce the possibility of cross contamination, specimens should be collected aseptically, with single-use devices. The fresh specimens should be shipped on ice for next day delivery; however, certain specimen types may be frozen or formalin-fixed prior to submission. Always contact the diagnostic laboratory to determine how to collect and ship the sample.
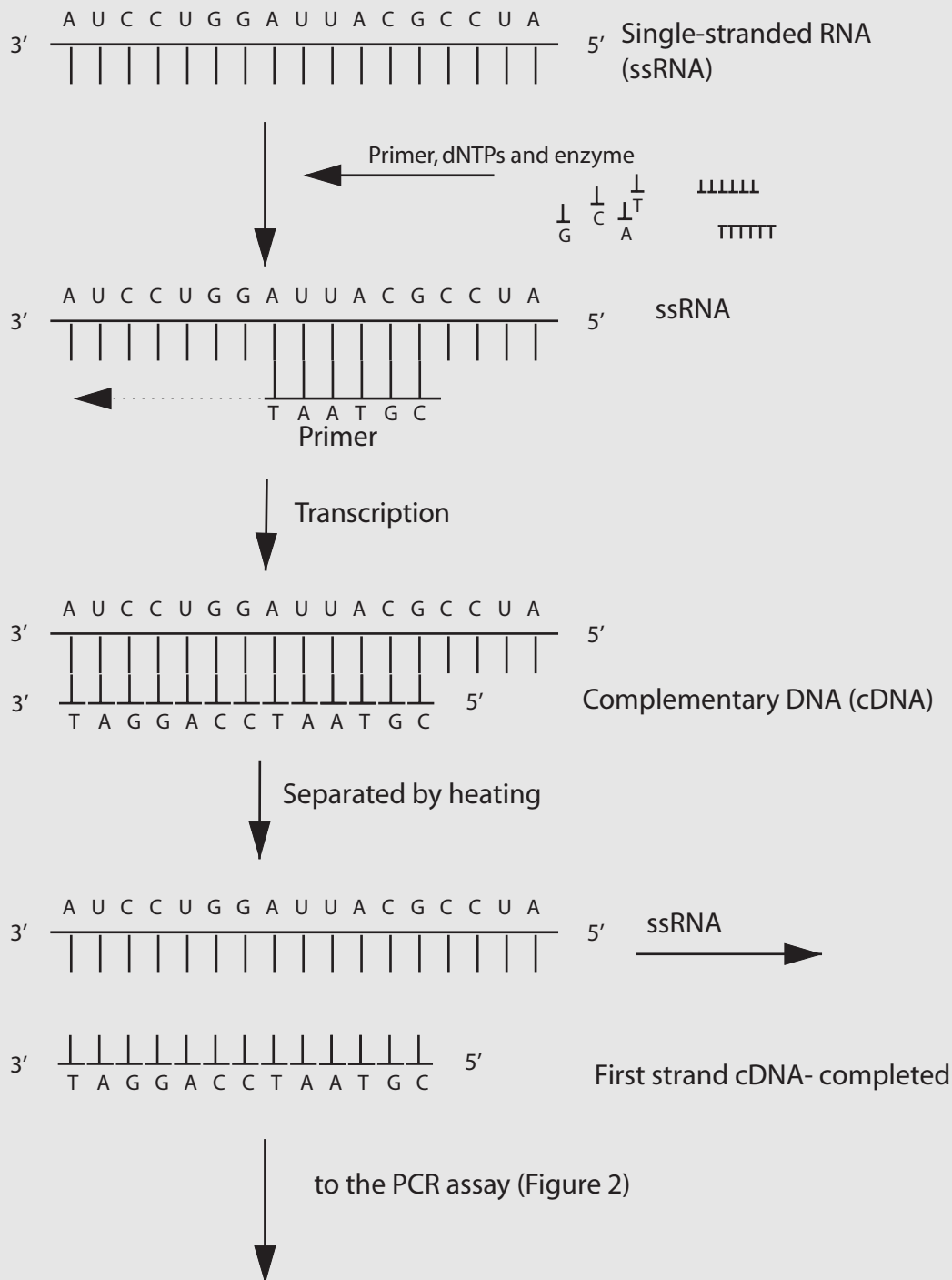
The PCR assay

PCR is used to amplify a specific DNA fragment that is between 150–1000 base pairs long. This DNA fragment is called the "target sequence." After the sample arrives at the diagnostic laboratory, the nucleic acid is first extracted. Extraction, the first step for all specimen types, removes excess protein and inhibitory substances, leaving pure, full-length DNA or RNA for the PCR assay.

Since the starting nucleic acid must be DNA, the isolated DNA from bacteria, parasites, or DNA viruses can be used directly. RNA from RNA viruses must first undergo reverse transcription (RT), an enzymatic process that converts the RNA into cDNA (a DNA strand complementary to the RNA), allowing the target sequence to be amplified (Figure 1).

The PCR assay itself is divided in three distinct stages:

- **Denaturation.** Occurs when DNA is heated to 95°C (Figure 2a). This causes the double-stranded DNA to "unzip," creating two single strands of DNA.
- **Annealing**. The temperature is reduced to 50°C to allow the primers to anneal or bind to the single-stranded DNA target (Figure 2b). Properly designed primers will bind only to the target region of the sequence, yielding an assay with high specificity.
- **Elongation**. The target sequence, primers, free-dNTPs, and Taq polymerase are heated to 72°C for the elongation step (Figure 2c).

Figure 1

A U C C U G G A U U A C G C C U A Single-stranded RNA (ssRNA)
3' 5'

Primer, dNTPs and enzyme

A U C C U G G A U U A C G C C U A ssRNA
3' 5'

T A A T G C
Primer

Transcription

A U C C U G G A U U A C G C C U A
3' 5'

3' 5' Complementary DNA (cDNA)
T A G G A C C T A A T G C

Separated by heating

A U C C U G G A U U A C G C C U A
3' 5' ssRNA

3' 5' First strand cDNA- completed
T A G G A C C T A A T G C

to the PCR assay (Figure 2)

Schematic of reverse transcription (RT). Primers specific for a region of mRNA bind to the single strand. Free-dNTPs are incorporated onto the primer with the use of the reverse transcriptase enzyme. The product of the RT reaction is a single-stranded cDNA, now ready to be amplified in the PCR assay.

In elongation, the free-dNTPs are paired up with the target sequence and cemented together by the Taq DNA polymerase to form a new double-stranded DNA molecule.

These three steps constitute one cycle of amplification. Denaturation, annealing, and elongation are repeated over and over on the auto-mated thermal cycler to amplify the DNA. After 30–40 cycles (2–3 hours), the initial DNA has been geometrically amplified (Figure 2c), providing billions of DNA copies (PCR product) from just one copy of target DNA. The geometric amplification is the sensitivity component of the assay.
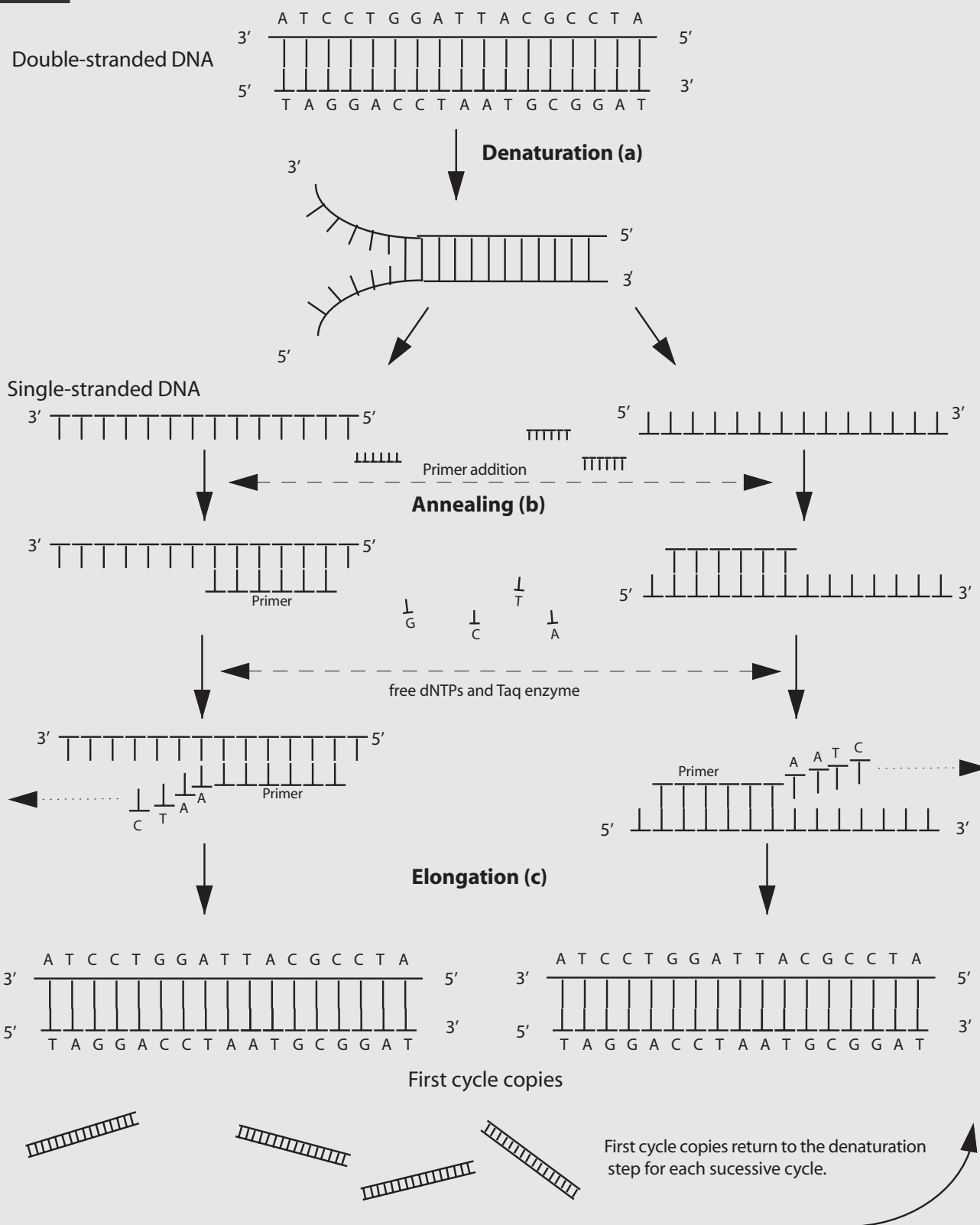
Schematic of the polymerase chain reaction (PCR) assay. (a) Double-stranded DNA is separated into two single-stranded pieces by denaturation. (b) Primers bind to specific regions of the target DNA strand. (c) The single-stranded DNA is elongated by the enzymatic incorporation of free-dNTPs. The double-stranded PCR products become target DNA for each consecutive cycle. After one cycle, the assay yields two copies for each copy of initial DNA. After two cycles, the yield is four copies. After three cycles, the assay yields eight copies. This continues for 20–30 cycles, yielding billions of copies of the original DNA fragment.

Gel electrophoresis

The amplified product is then detected by agarose gel electrophoresis. Gel electrophoresis is a technique by which one can sort DNA fragments of different sizes. Electric current is passed through the gel, which causes the DNA to migrate. Shorter fragments will travel farther than longer fragments.

Fragments of known size are placed into the first lane and a fraction of the PCR product is placed into consecutive lanes. Then, current is applied and the DNA is allowed to migrate. The gel is stained with a fluorescent dye (ethidium bromide) which will only bind to the DNA present in the agarose gel. The gel is visualized with an ultraviolet light and documented in a photograph (Figure 3). The size of the amplified PCR product is determined and compared to standard controls. Other detection techniques are being developed that will result in a colormetric reaction, reducing the time needed to complete the assay and allowing quantification of the amount of PCR product.

Currently, there are PCR assays for both porcine viral and bacterial agents. Assays are currently available to detect the following viruses:

- PRRSV,[5]
- transmissible gastroenteritis virus (TGEV),[6]
- porcine respiratory coronavirus (PCRV),[7] and
- pseudorabies virus (PRV).[8]

Some of the bacterial pathogens that can be detected by PCR include:

- *Lawsonia intracellularis*,
- *Serpulina hyodysenteriae*,
- *Salmonella* spp, and
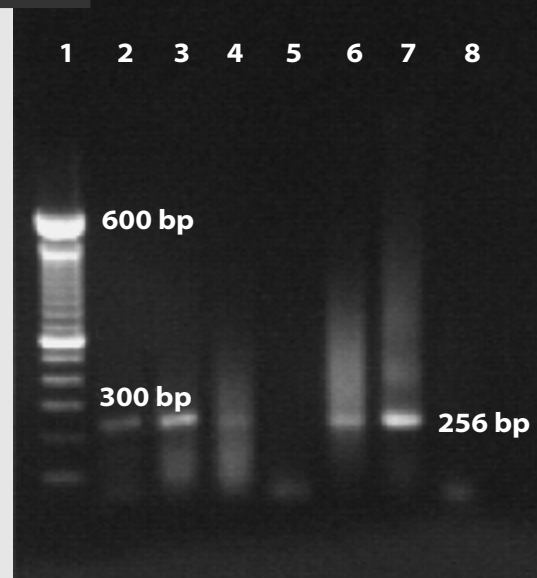- *Mycoplasma* spp.[9,10]

Many more PCR assays are sure to be made available for pathogenic organisms and their subspecies whose importance warrants the development of specific assays.

The improvement in the three Ss—sensitivity, specificity, and speed—offered by PCR have made it an invaluable tool in diagnostic laboratories. Although PCR offers many benefits, it is important to use it appropriately. PCR is not recommended, for example, as a herd screening tool because of its expense. Its use should be reserved for situations in which it is essential to know whether a specific pathogen is present—e.g., boar testing or testing for persistently infected animals.[11] As with other technological advances, PCR testing will become more affordable and routine as its use evolves.

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Figure 3

Agarose gel electrophoresis is a common method of determining the size of PCR products. This figure is an example of a PRRSV PCR electrophoresis gel. A single band at 256 base pairs indicates a positive test result. Lane 1 is a 100 bp ladder, used to estimate the size of the PCR products. Lanes 2–4 and 6 are positive field isolates. Lane 5 is a negative field sample. Lane 7 is the positive control and Lane 8 is the negative control sample.