# Rapid Detection of Hendra Virus Using Magnetic Particles and Quantum Dots

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Hendra virus (HeV) is a new and deadly emerging pathogen, which was first detected after an outbreak in 1994 in Australia resulting in the death of horses and humans.[1] Since 1994, there have been more than 30 outbreaks with annual occurrence since 2006.[2] The serious damage to livestock and the public health threat have attracted the attention of the Australian government and the general public.[1,2] The early detection of Hendra virus is essential for any successful countermeasure strategy. In an emergency infectious disease response, the rapid detection and diagnosis of the causative agent(s) via a highly sensitive platform are of paramount importance in mitigating the damage of an outbreak.[3] In an ideal scenario, the detection should be performed at the outbreak site (point-of-care testing[4]) using a portable device (e.g., microfluidic platform).[5,6] In this context, currently there is no commercially available device for the detection of Hendra virus.[2] This work describes for the first time a protocol for Hendra Virus detection involving functional particles conjugated with Hendra virus-specific antibodies. The virus is revealed by the simultaneous presence of magnetic and luminescent functional particles and demonstrates proof-of-concept for the development of a fast and portable Hendra virus biosensor.

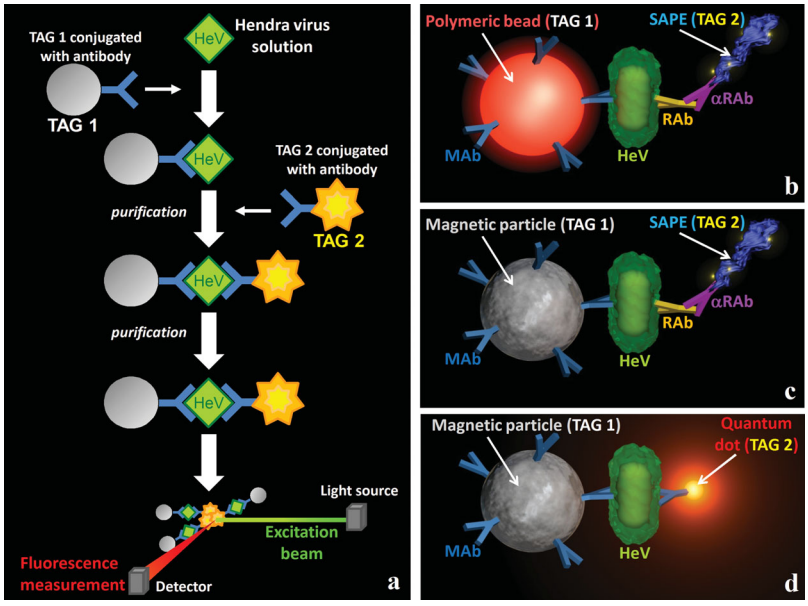


**Figure 1.** a) General scheme of the co-localization method used to detect Hendra virus. The virus binds two different functional tags (TAG 1 and TAG 2); after purification, the fluorescence measurement reveals the presence of the virus. b) The first system: The virus is co-localized by the simultaneous emission of the two fluorescent tags: the MAb-conjugated fluorescent polymeric beads (TAG 1) and the SAPE fluorescent macromolecular complex (TAG 2). c) The second system: The virus is co-localized by MAb-conjugated magnetic particles (TAG 1) and by the SAPE complex (TAG 2). The fluorescence detected after magnetic separation determines the presence of the virus. d) The third system: The virus is co-localized by magnetic particles (TAG 1) and luminescent quantum dots (TAG 2), both of them conjugated with MAb antibodies, specific for Hendra virus (HeV).

The strategy used in this study is called "co-localization". This method requires at least two tags for the detection process. If the tags are simultaneously bound to the bioanalyte, then a signal is detected. Chang et al. first proposed this approach in 2008 for *Dengue* virus detection; they employed the simultaneous binding of magnetic particles and luminescent polymeric beads to the bio-analyte.[7] This principle is also utilized in various immunoassays, e.g. in the Luminex Assay,[8] which is the current state-of-the-art method for the detection of Hendra virus. It employs luminescent polymeric beads and a luminescent protein. However, the Luminex Assay requires a non-portable instrument and therefore point-of-care (outbreak location) detection is currently not possible and this limitation represents a serious weakness.

Recently, the monoclonal antibody "IgG1 m102.4" (MAb) for Hendra virus has been synthesized[9] and the three proposed systems take advantage of the specific antigen-antibody interaction.[10,11,12] The method proposed in this study also employs two different functional tags conjugated with MAb antibodies and is illustrated schematically in **Figure 1**a. The

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first system utilizes the same basic approach as the Luminex Assay (luminescent bead–virus–luminescent protein), while the second (magnetic bead–virus–luminescent protein) and third (magnetic bead –virus–quantum dots) assay methods are stepwise modifications to the luminescent protein Assay. The first system is used as a benchmark for evaluating the performance of the second and third approaches.

In the Luminex Assay, fluorescent polymeric beads (PBs, emission around 708 nm) are conjugated to MAb antibodies, which selectively bind the Hendra virus (HeV). The virus surface is also bound by another type of Hendra virus selective antibody (RAb). RAb antibodies are then attached by biotinylated secondary antibodies ($\alpha$RAb) reactive towards a fragment of the previous antibodies. A macromolecular complex (SAPE) composed of streptavidin and R-phycoerythrin (a fluorescent protein, emission around 575 nm) binds the biotin fragment of the secondary antibody, taking advantage of the very stable interaction between streptavidin and biotin. These reactions lead to the first system studied (named PBs–< HeV >–>–SAPE, where the symbol "–<" represents an antibody, see Figure 1b). In the second system (named MPs–< HeV >–>–SAPE, see Figure 1c) the PBs have been substituted by magnetic particles (MPs), bound to MAb antibodies, which allows the magnetic separation, concentration and positioning of the bioanalytes at a specific location. In the third system (named MPs–< HeV >–QDs, see Figure 1d) the magnetic particles are still present but the SAPE fluorescent probes are substituted with quantum dots (QDs, emission around 642 nm) conjugated to human monoclonal antibodies.

Quantum dots have been chosen mainly to simplify the detection process and to facilitate development of a portable assay. Indeed, compared to the previous two systems, this third method reduces the number of reactions and washing steps needed, leading to a faster and easier detection. Furthermore, quantum dots offer higher brightness. The brightness is defined as the product of the molar absorption coefficient at the excitation wavelength and the fluorescence quantum yield;[13] it is used as a measure for the intensity of the fluorescence signal obtainable upon excitation at a specific wavelength. An attractive feature of QDs is that even if the QDs quantum yield is lower than some organic dyes, the molar absorption coefficients of QDs are generally much larger than those of organic dyes, leading to higher brightness.[13,14]

Bioconjugation has been carried out using EDC coupling of the primary amino groups (on the lysine side chains and at the N-terminus of the antibody polypeptide chains) to the carboxylic acid groups present on the microbead surface. The amide bond formation has been promoted using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo-(n-hydroxysulfosuccinimide) (S-NHS) as coupling agents (see Supporting Information, Figure S1).[15,16] Several variables influence the conjugation reaction, such as the particle size, the antibody concentration and the EDC + S-NHS concentration.

The conjugation reaction has been characterized using fluorescence spectroscopy through the following steps: 1) a reaction between biotinylated secondary antibodies ($\alpha$MAb) and the immobilized antibodies (Mab) was performed; 2) the SAPE luminescent complex was attached to the biotinylated $\alpha$MAb; 3) the sample was investigated with a spectrofluorimeter to detect the luminescent signal (see Supporting Information, Figure S2). The background fluorescence of the proposed system is negligible; only the samples prepared with the correct sequence of reagents exhibit fluorescence (see Supporting Information, Figure S3). The same detection method has also

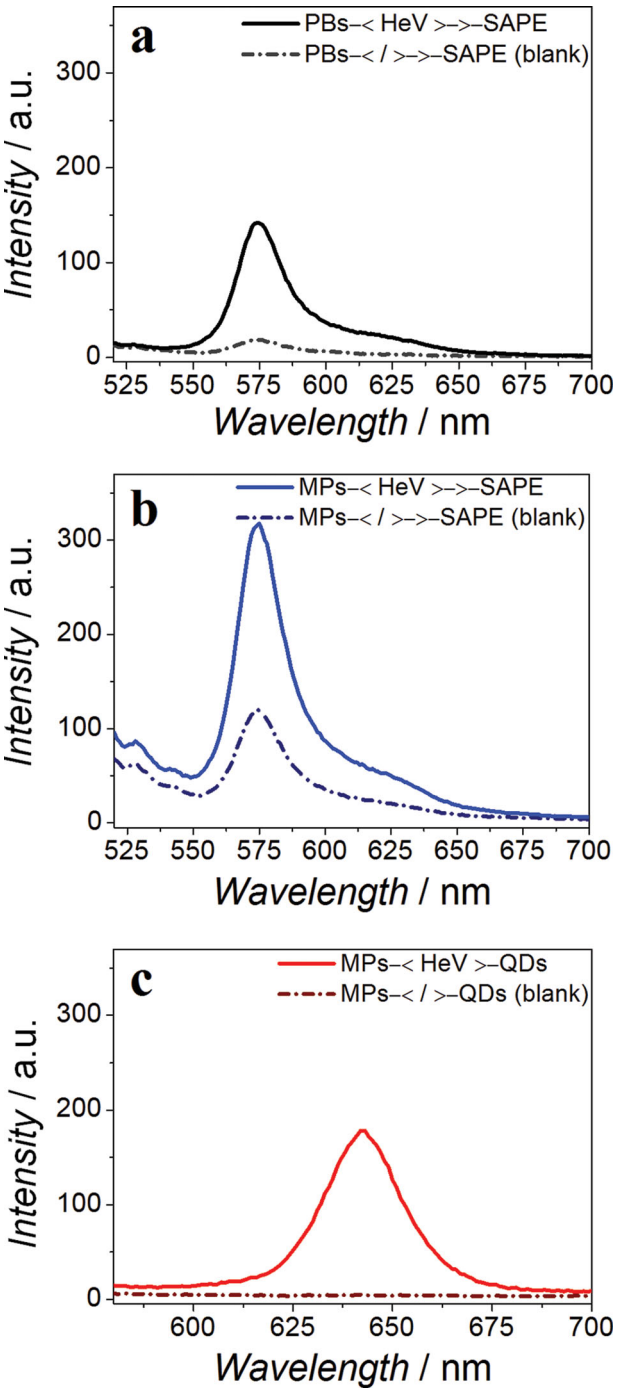


**Figure 2.** Fluorescence spectra of one sample for each system PBs–< HeV >–>–SAPE, MPs–< HeV >–>–SAPE, MPs–< HeV >–QDs and the respective blanks PBs–</ >–>–SAPE, MPs–</ >–>–SAPE, MPs–</ >–QDs. Excitation wavelength = 498 nm. Magnetic particle size = 240 nm, quantum dot size = 9 nm.



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been used to optimize the above-mentioned variables during the synthesis of antibody-coupled magnetic particles and antibody-coupled quantum dots (see Supporting Information, Figure S4). The intensity of the fluorescence signal, the colloidal stability, and the reproducibility of the samples are the output parameters utilized to rank the samples and then optimize the above-mentioned variables. The optimized bioconjugated particles have been used to detect HeV, producing the three different configurations described above (see Figure 1).

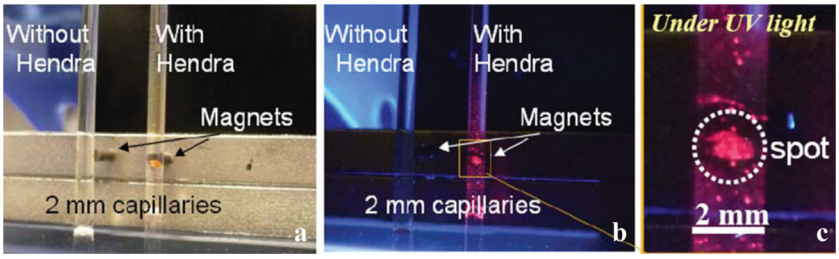


**Figure 3.** Pictures of two glass capillaries (2 mm diameter) with MAb-conjugated 240 nm magnetic particles (MPs–<) and 9 nm quantum dots (QDs–<). Left; blank sample (no HeV). Right; positive sample (with HeV). The samples have been inserted into two capillaries placed near a magnet to demonstrate the ability to concentrate the particles and thus enhance the signal. a) Daylight illumination. b) Illumination at 365 nm. c) Magnification of the previous image, showing the 1.5 mm luminescent spot.

In order to confirm the reproducibility of the method, six samples have been synthesized for each system: three replicates with HeV (indicated as TAG1-HeV-TAG2) and three replicates without HeV (blank samples, indicated as TAG1-/-TAG2). The sample fluorescence has been measured and the resulting spectra are shown in **Figure 2**. Control tests and fluorescence spectra of the intermediate products have been collected and are shown in the Supporting Information, Figure S5. The coefficient of variation[17] of the fluorescent signal was used to evaluate the reproducibility of the system; the lower the coefficient of variation the more reproducible the system. The signal-to-noise-ratio[18] was used to evaluate the reliability of the detection method.

In the first system (HeV detected using PBs and SAPE, see Figure 2a) the coefficient of variation of the fluorescence maximum intensity is 15% and the signal signal-to-noise ratio is approximately 7. The configuration with MPs and SAPE (see Figure 2b) has a coefficient of variation of 5%, while the signal-to-noise ratio is 2. Hence, use of magnetic separation increases the reproducibility of the assay. Replacing the protein complex SAPE with QDs (third system), increases the coefficient of variation to 28% while the signal-to-noise ratio increases drastically to 36 (measured at 642 nm, see Figure 2c). The reproducibility of the signal is decreased due to the partial precipitation of QDs; however, the luminescent particles provide a superior signal-to-noise ratio (500% improvement with respect to the first system). This reduces the rate of "false positives"[19] to almost zero).

To demonstrate the portability of the assay protocol, the samples MPs–</ >–QDs (blank) and MPs–< HeV >–QDs have been introduced into two glass capillaries (2 mm diameter) placed near two magnets (2 mm diameter) and illuminated by a low power UV lamp (15 W, 365 nm). The contrast between positive and blank samples can be directly discerned by the naked eye, as evident from the photographs in **Figure 3**. Confinement of HeV labelled quantum dots produces a 2 mm bright luminescent red spot.

In summary, HeV is a new emerging pathogen posing a serious threat to livestock (over 90% mortality for infected horses) and humans (over 50% mortality).[1,20] The current HeV detection method is based on co-localization using luminescent beads and proteins. However, this strategy does not allow for rapid detection as too many steps are needed and the detection procedure requires laboratory based instrumentation. To develop a straightforward detection method, two improvements have been proposed and tested. A key advantage of the modifications is the ability to prepare in advance and store the antibody-conjugated MPs and QDs. These antibody-coupled particles can be introduced into a portable device (e.g. microfluidic circuit) allowing for detection at the point-of-care. The third approach, which utilizes quantum dot based fluorescence detection, is the simplest method with the least number of reactions and washing steps needed. While further optimization of the system is required to maximize the sensitivity of the assay, it is evident that the combination of magnetic separation and QD based luminescence detection should be applicable to a wide range of antibody-antigen assays. In particular, the simplicity and robustness of the current protocol lends itself to development of a portable biosensor for Hendra virus.

## Experimental Section

*Materials*: Carboxylated polymeric beads "MultiPlex® Microspheres" (PBs, 5.6 µm in diameter) were purchased from Fisher Biotech Australia. Carboxylated magnetic particles "SPHERO™" (MPs, various diameters from 0.24 to 9.97 µm, see Supporting Information, Figure S6) were purchased from Spherotech. Streptavidin/R-phycoerythrin conjugate (SAPE) was purchased from Invitrogen. Biotinylated secondary antibodies, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo- (n-hydroxysulfosuccinimide) (S-NHS) were purchased from Thermo Scientific. Carboxylated quantum dots (QDs, diameters of 9 and 7 nm, emission wavelength respectively of 645 and 618 nm) were synthesized by standard chemical procedures.[14] Inactivated Hendra virus (HeV) and Rabbit anti-HeV antibody (RAb) were prepared in-house at CSIRO AAHL.[10,11] IgG1 m102.4 antibody (MAb) was kindly provided by Dr. C. Broder from the Uniformed Service University (USA).

*Antibody Immobilization Reaction*: The carboxylated particles were activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo- (n-hydroxysulfosuccinimide) (S-NHS) as coupling agents, following the protocol described by Hermanson.[15] The MAb antibodies were then immobilized on the activated particles through the formation of an amide bond and the final products were washed 3 times.

*Fluorescence Measurements*: Fluorescence spectra were acquired using a Cary Eclipse Spectrofluorimeter. All the measurements were performed with an excitation wavelength of 498 nm.

*Variable Optimization*: Particle size, antibody concentration, and EDC + S-NHS concentration were optimized for both magnetic particles and quantum dots. Within the investigated variable range, the best detection performance was found using 0.24 µm magnetic particles reacted with EDC (1 mg/ml), S-NHS (1 mg/ml) and MAb antibodies (60 µg/ml) for the corresponding bioconjugation reaction, and 9 nm quantum dots (645 nm emission) reacted with EDC (10 mg/ml), S-NHS (10 mg/ml)

and MAb antibodies (7 μg/ml) for the corresponding bioconjugation reaction (see Supporting Information, Figure S4).

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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