Biotechnological Communication

Biosci. Biotech. Res. Comm. 9(3): 349-356 (2016)

BBRC Bioscience Biotechnology Research Communications

Recent advancements in molecular detection of *Vibrio* species in aquatic animals: A review

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ABSTRACT

Vibriosis is one of a major problem in aquaculture sector causes by *Vibrio* species and gives bad impact on economy and social development. In addition, number of the infected people with vibriosis is increasing every year through consuming of contaminated fishes and other aquatic animals. To overcome this problem, researchers continuously develop new methods for identification and detection of *Vibrio* species. Those methods can be categorized based on the principle applied; conventional culture method, antigen-antibody based assay, nucleic acid amplification technology and lateral flow dipstick. The conventional culture method is a basis in identification of *Vibrio* but it is time consuming and requires skilled personnel. The nucleic acid amplification technology such as isothermal amplification assay in a combination of lateral flow dipstick is widely used since these assays offer rapid, easy to handle and high sensitivity and specificity detection methods. This paper also reviews and describes the available application and limitations of the studies involving *Vibrio* detection methods of aquaculture field. As a conclusion, the development of new technologies is very important to improve the detection of *Vibrio* species as well as increasing the number of food production to meet human demands.

KEY WORDS: ISOTHERMAL AMPLIFICATION, LAMP, LATERAL FLOW DIPSTICK, NUCLEIC ACID AMPLIFICATION, PCR, *VIBRIO*

INTRODUCTION

Nowadays, aquaculture becomes an important food production sector especially from fishes, molluscs, crustaceans and other aquatic animals. According to FAO (2013), 128 million tons of fishes, molluscs and crustaceans were provided for human food. The number of food production will be increasing every year due to high demand for food. In addition, aquaculture is a significant socio-economic activity through employment, income generation,

ARTICLE INFORMATION:

**Corresponding Author: salwany@upm.edu.my*
Received 18th Aug, 2016
Accepted after revision 26th Sep, 2016
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007
Thomson Reuters ISI ESC and Crossref Indexed Journal
NAAS Journal Score 2015: 3.48 Cosmos IF : 4.006

Online Contents Available at: http//www.bbrc.in/

domestic and international trade, and provide livelihoods especially for rural communities (Edward et al., 2002). Unfortunately, the diseases problem of aquaculture sector is increasing every years caused by bacteria, viruses, fungi, parasites and other emerging pathogens (Adams and Thompson, 2011). This problem also effects the economic and social development worldwide.

Vibriosis is one of a major problem in aquaculture sector and caused outbreaks of aquatic animal diseases. It leads to significant mortality in aquatic animals, loss of food and severe economic losses (Buller, 2014; Gauthier, 2015).

Besides, this bacterium can infect human through consumption of raw or undercooked fish and other aquatic animals. Outbreaks of vibriosis have been reported worldwide caused by *Vibrio alginolyticus, V. parahaemolyticus, V. harveyi, V. anguillarum, V. vulnificus* and other *Vibrio* species in various countries, including United State, Canada, China, Taiwan, India and Japan (Chiou et al., 2000; Alam et al., 2003; Deepanjali et al., 2005; Xie et al., 2005; CDC, 2013). Over 80 000 cases and 100 deaths caused by vibriosis were reported in United State every year (Scallan et al., 2011). According to CDC, the number of cases in U.S. was increasing 75% in 2013 compared to 2006 until 2008 with majority of cases caused by *V. parahaemolyticus* and *V. vulnificus* (FoodNet, 2014).

Vibrio is a gram negative bacterium from family *Vibrionaceae*, rod shape and undergoes facultative anaerobic respiration. Naturally *Vibrio* found in estuarine, marine environments, normal flora of organisms and also can be pathogenic (Wang et al., 2009; Haldar et al., 2011; Chatterjee and Haldar, 2012). Usually, there are no specific symptoms of infection of vibriosis observed in marine life. The symptoms are similar with other bacteria diseases starting with lethargy and loss of appetite. The skin becomes discolored, red and boil-like sores may appear on the skin surface. As a disease progress, erythema or bloody blotches can be found around the mouth and fins. The gut and rectum can be bloody and filled with fluid if an early treatment not be given (Wang et al., 2009). Thus, all these symptoms are not proof of a vibriosis and require a further isolation and identification of bacteria.

Therefore, culture method has been developed for the identification of bacteria species in aquatic animal and marine environment. The bacteria are allowed for multiplying in culture media under controlled laboratory conditions and further identified using gram staining and biochemical tests. However, this method is tedious, time consuming and requires skilled personnel to pick right colonies of a plate. In addition, culture method unable to differentiate species levels due to similar morphology such as *V. cholerae, V. alginolyticus* and *V. fluvialis* which appeared yellow color on agar plates (Oxoid, UK).

To overcome those limitations, the antigen-antibody based assay was developed to test presence of specific antibody or antigen in aquatic samples. A specific antibody is used for recognition of antigen from pathogenic bacteria. Even though this assay is rapid and easy to perform, but it is unable to differentiate *Vibrio* species because they have same antigens (Chen et al., 1992). The molecular approaches based on synthesis of nucleic acid were developed for producing more sensitive and specific assays such as polymerase chain reaction (PCR), real-time PCR, multiplex PCR, reverse-transcriptase PCR, touchdown PCR and so on. However, those assays required expensive thermocycler machine to amplify the nucleic acid based on three different temperatures.

Thus, a novel isothermal amplification assay was developed in early 1990s as an alternative to the PCR assay. The amplification of nucleic acid can be performed in a single temperature (isothermal condition) which mimics *in vivo* synthesis of DNA. The isothermal amplification assay can be performed without the need for a thermocycling apparatus. In addition, the isothermal amplification assays also more sensitive and specific, rapid and easy to perform. However, the detection and visualization of isothermal products still requires conventional agarose gel electrophoresis system. This agarose gel electrophoresis is laborious, requires special equipment, time consuming and must be operated by skilled personnel. Thus, an alternative method, a lateral flow dipstick is developed for the detection of isothermal products. Based on previous study, the lateral flow dipstick is rapid (within five to ten mins), easy to perform, does not require special equipment and can be visualized using naked eyes (Wain and Hosoglu, 2008; Mugasa et al., 2009).

CONVENTIONAL CULTURE METHODS

Culture method is a gold standard for isolation of bacteria by using enrichment and selective medium. Basically, tryptic soy broth (TSB) is used to enrich amount of *Vibrio* from samples. *Vibrio* can grow in different environments due to its ability to live in saltwater, freshwater and living organisms. *Vibrio* grows well at pH 7 to 9, from neutral to alkaline condition. It also able to grow in high concentration of sodium chloride (NaCl) due to it character halophilic. A sodium chloride (NaCl) is added with the TSB when samples were collected from saltwater. In laboratory, a halophilism test was performed with different concentration of NaCl (0 to 10%) and *V. alginolyticus* requires at least 3% NaCl and can tolerate until 10% NaCl for growth (Gomathi et al., 2013).

Thiosulfate citrate bile salts-sucrose (TCBS) agar is a common selective media used for isolation of *Vibrio* species (Jones et al., 2012). In order to perform a bacteria culture on the agar plate requires a lot of time to allow optimum growth of bacteria colonies. For example, bac-

Table 1: Typical morphology of *Vibrio* colonies on TCBS agar

Organisms	Size of colonies	Color of colonies
Vibrio parahaemolyticus	3-5 mm diameter	Blue colonies with green centers
Vibrio alginolyticus	3-5 mm diameter	Yellow
Vibrio cholera	2-3 mm diameter	Yellow and flat colonies
Vibrio vulnificus/ Vibrio mimicus	2-3 mm diameter	Blue green
Vibrio harveyi	2-3 mm diameter	Gray to bluish green colonies
Vibrio fluvialis	2-3 mm diameter	Yellow

teria are incubated at 30°C for 16-24 hours when isolated from environmental samples, while bacteria are incubated at 35°C for 24 hours when isolated from other samples (Oxoid, UK). Typical colonies of *Vibrio* can be varying in terms of size and color of colonies (showed in Table 1).

The biochemical profile of *Vibrio* species can be determined using API 20E diagnostic strip (bioMerieux, Durham, NC). The API 20E test strip has 20 miniature biochemical tests of different medium; Citrate (CIT), Voges-Proskauer (VP), Urease (URE), hydrogen sulfide (H_2S), amino acid decarboxylations (ADH), gelatin hydrolysis (GEL) and others. Bacteria are mixed with two percents of NaCl as the suspension solution, added to the API strip and incubated at 37°C for 18-24 hours. After that, changes of the color reaction were read and converted to a seven digit code known as Analytical Profile Index (API). The codes are matched with the manufacturer's database and give the bacteria identification as a genus and species. According to Jones (2012), API 20E test was able to identified 41.8% of *V. parahaemolyticus* from oysters isolates and 54.6% of *V. parahaemolyticus* from clinical isolates. The API codes were misidentified several bacteria isolate such as *V. vulnificus, Aeromonas hydrophila, V. fluvialis, V.cholerae* and *V. mimicus* (Jones et al., 2012).

However, these conventional methods have disadvantages such as less specific, tedious, require skilled personnel and cannot differentiate between *Vibrio* species, especially *V. parahaemolyticus* and *V. alginolyticus* which are biochemically similar (Mustapha et al., 2013).

ANTIGEN-ANTIBODY BASED ASSAY

Vibrio species possesses three antigenic components; heat-labile K-antigen (capsular polysaccharide), heat-stable somatic O-antigen (lipopolysaccharide) and H-antigen (flagellar). Those antigenic components of *Vibrio* can be determined via agglutination tests where the bacteria culture are reacted with specific antiserum to formed clumps or agglutinate particles. To determine the O-antigens, the bacteria should be heated at boiling temperature for one to two hours. This step is important to removed K-antigen from *Vibrio* since K-antigen masked the O-antigen.

The *Mono-aqua* test kit (BIONOR, Tamar Laboratory Supplies Ltd., Israel) is a commercial agglutination tests for rapid preliminaries screening of pathogens such as *V. anguillarum* and *V. salmonicida*. The principle used is a mono-disperse particles coated with specific antibodies will be reacted with fish pathogenic bacteria (antigen) and form a visible granular particle agglutination pattern. This kit is rapid (can be performed in 30 minutes) and inexpensive. However, the samples have to be cultured overnight before testing and make this test tedious and time consuming (Romalde et al., 1995). Also, *Vibrio* species are serologically identical based on a study reported that most of the *Vibrio* species share H-antigen at 52-kDa protein including *V. parahaemolyticus, V. alginolyticus, V. anguillarum* and *V. ordalii* (Chen et al., 1992).

An *aqua-rapid* and *aqua-EIA* test kits were developed as a modification of previous kit based on Enzyme Immunoassay (EIA) principle. EIA is also known as enzyme-linked immunosorbent assay (ELISA), combination of the antibody binding with certain enzyme for the detection and quantification of antigen (pathogenic bacteria) (Lequin, 2005). By using the EIA principle, both kits can be used for direct identification of fish pathogens without needs of culture. Thus, these kits are rapid, easy to performed and suitable to be used at farm site. The *aquaEIA* test kit is using semiautomated system, while *aqua-rapid* using manual system.

The dot immunoassay method is one of the antigen-antibody based assay that has been used to detect *V. alginolyticus* and *A. hydrophila* isolated from shrimps and fishes. The bacteria culture is diluted before spotted onto the nitrocellulose paper and allow reacting with antiserum raised against *V. alginolyticus* and *A. hydrophila*. Even though this method is simple, but it is less specific due to cross-reaction of *V. alginolyticus* antiserum with *V. parahaemolyticus, V. harveyi* and *V. anguillarum* (Mishra, 1998).

NUCLEIC ACID-BASED ASSAY

Molecular diagnostics involving nucleic acid amplification is the most common methods used for the detection and identification of infectious diseases. Polymer-

ase chain reaction (PCR) is a main technique used for nucleic acid amplification. PCR provides a rapid, high sensitivity and specificity compared to the conventional culture methods for differentiation and identification of species. It is also able to identify housekeeping genes and different virulence genes among *Vibrio* species. In addition, the PCR assay is able to simultaneous detection of multiple pathogens in a single reaction known as multiplex PCR assay. The PCR assay is based on the amplification of nucleic acids using specific primer and DNA polymerase at three different temperatures; denaturation, annealing and extension that repeated in a number of cycles until certain amount of DNA obtained. Thus, the PCR assay requires a thermal cycler to complete the amplification process.

Researchers were performed extensive studies using PCR assay for the identification and differentiation of *Vibrio* infected fishes, molluscs, crustaceans and other aquatic animals. Various genes were used in designing primers for specific amplification of target gene of *Vibrio*. A 16S rRNA gene is highly conserved among bacteria species based on hyper variable region that contains similar species-specific sequence which is useful for bacteria identification. In 2006, Bramhachari and Duey developed a PCR for detection of *V. harveyi* using 16S rRNA. Kim and Jeong (2001) was developing a PCR targeting the same 16S rRNA gene to identify pathogenic *V. vulnificus* in marine environments. Similarly, Yong in 2006 targeted gene for identification of bacteria from aquaculture systems.

A *toxR* gene is a virulence gene encodes for regulatory gene of toxin operon. A PCR for detection of *V. harveyi* targeting *toxR* gene was successfully developed with 100% specificity (Conejero and Hedreyda, 2003). In 2016, the *toxR* gene is found to be more specific compared to the *gyrB* gene in identification of *V. parahaemolyticus* in fish and coastal environment in Jordan (Alaboudi et al., 2016). *GyrB* gene is encodes for the B subunit protein of DNA *gyrase* (topoisomerase type II). Previous study was found the degree of homology of toxR gene between *V. parahaemolyticus* and *V. cholerae* was 52% (Kim et al., 1999).

Other virulent genes used for identification of *Vibrio* species were thermostable direct hemolysin (*tdh*), thermolabile hemolysin (*tlh*) and thermostable direct hemolysin-related hemolysin (*trh*) (Jones, 2012). Mohammed and Jerjees (2015) proved that the 12% and four percents of *V. parahaemolyticus* isolated from fresh shrimp have the *tdh* and *trh* genes, respectively. Other study revealed the *tdh* and *trh* genes were 98% homology between *V. alginolyticus* and *V. parahaemolyticus* isolated from shrimp (Gargouti et al., 2015). In 2016, Li and his friends found a unique gene for *V. parahaemolyticus*, $bla_{CARB-17}$ gene which has the lowest degree of similarity (78% homology) with *V. alginolyticus* compared with other virulence genes such as *tlh* (85%), *atpA* (97%) and *toxR* (86%) genes. The PCR assay targeting for $bla_{CARB-17}$ gene was produced 100% sensitivity and specificity (Li et al., 2016).

A multiplex PCR (mPCR) assay was used for simultaneous detection of more than one *Vibrio* species in a single test. Xie (2005) was developing the mPCR assay for detection of seven virulence genes targeted for *V. parahaemolyticus* and *V. alginolyticus* from shrimp, fish and seawater in Guangdong Coast, China (*tlh, trh, tdh, toxR, toxRS, ctxA* and *VPI*). This study found that the *V. alginolyticus, V. parahaemolyticus* and *V. cholerae* possess homology using *toxR, tlh* and *VPI* genes. In 2011, Izumiya was developed a highly sensitive and specific mPCR assay for the detection of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* from seawater sample using dnaJ gene. Similarly, Xu in 2014 was targeted dnaJ gene for detection of five *Vibrio* species*; V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. cholerae* and *V. mimicus* from seafood samples.

In addition, the 16S rRNA gene was included in the mPCR assay as a *Vibrio* species control to avoid any doubt regarding target of *V. parahaemolyticus, V. mimicus, V. vulnificus* and *V. cholerae* from shrimp and crab samples (Amin and Salem, 2012). The 16S rRNA gene also used as an internal amplification control (IAC) in the mPCR assay targeting *V. alginolyticus, V. parahaemolyticus, V. vulnificus* and *V. cholerae* (Wei et al., 2014). The internal amplification control is a mandatory for diagnostic using PCR assay. IAC is used for validation of negative result whether it is truly negative or due to malfunction of thermal cycler, incorrect PCR mixture or presence of inhibitory substances in the sample (Hoorfar et al., 2003).

Based on previous studies, the PCR assay is highly sensitive and specific, able to simultaneous detection of many pathogens in a single tube and successful detect *Vibrio* species. However, this assay requires expensive equipment, time consuming and requires trained personnel. Based on the WHO guidelines, a develop diagnostic device must be affordable, sensitive, specific, user friendly, rapid and robust, equipment free and deliverable to end used (ASSURED) (Mabey et al., 2004). Thus, an alternative platform known as isothermal amplification assay was developed in order to fulfill this guideline.

ISOTHERMAL AMPLIFICATION ASSAY

Isothermal amplification techniques have been developed since early 1990s as an alternative to the polymerase chain reaction (PCR) assay. This technique is used to amplify DNA, RNA, cells and proteins. The isothermal amplification assay can be performed at one temperature rather than the PCR assay which required three different temperatures (Notomi et al., 2000; Chow

et al., 2008; Fang et al., 2010). Therefore, the isothermal amplification can be performed using simple heating devices (e.g., water bath or heating block) without the need of thermocycling apparatus. In addition, the isothermal product can be directly visualized by naked eyes on the form of turbidity or fluorescence, eliminating the need for electrophoresis system as used in the PCR assay. Thus, the isothermal amplification are rapid, user friendly, high sensitivity and specificity and can be performed without expensive equipment.

Nowadays, several types of isothermal amplification technique were developed for the detection of pathogenic bacteria such as loop-mediated amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), helicase dependent amplification (HDA), rolling circle amplification (RCA) and transcription mediated amplification (TMA) assays (Compton, 1991; Walker et al., 1992; Fire and Xu, 1995; Notomi et al., 2000; Vincent et al., 2004). Each isothermal assay had their own mechanisms to promote a next cycle of DNA synthesis. Differ with the PCR assay that used heat denaturation of double stranded DNA to initiate next cycles of DNA synthesis; the isothermal assays use various accessory proteins to open the double stranded DNA (Gill and Ghaemi 2008).

Loop-mediated amplification (LAMP) assay is the most popular isothermal amplification technique used in aquaculture field. LAMP assay was developed by Notomi et al. (2000) for the identification of Hepatitis B virus. This assay was designed based on four to six primers to recognize six or eight distinct sequences on the template DNA. The nucleic acid is extended by the *Bst polymerase* to synthesis new DNA strands. The entire LAMP reaction can be performed in simple heating devices at 60 to 65°C for less than one hour. The amplified products will be mixture of stem-loop DNAs with various length and cauliflower-like structures with multiple loops (Notomi et al., 2000; Mori and Notomi, 2009). The products can be identified by using agarose gel electrophoresis (produced multiple bands of different sizes from ~300bp), restriction enzyme digestion (such as *BamHI*, *HindIII* and others to produce two different bands) and Southern blot hybridization. Also, the LAMP products can be observed by naked eyes through usage of magnesium pyrophosphate, calcein, SYBR Green or other fluorescence dye (Prompamorn et al., 2011).

LAMP assay has been developed for the detection of *Vibrio* species such as *V. alginolyticus, V. harveyi* and *V. parahaemolyticus* from aquatic animals and environment (Cai et al., 2010, Di et al., 2015). The LAMP assay was developed for the detection of *V. alginolyticus* in mariculture fish using *gyrB* gene and it is ten-fold more sensitive than PCR assay (Cai et al., 2010). In addition, several studies revealed that the LAMP assay is superior to the PCR assay and conventional culture method for the detection of *V. parahaemolyticus* from environmental samples (Di et al., 2015; Kongrueng et al., 2015; Malcolm et al., 2015). A universal LAMP primer from conserved region of 16S rRNA was designed as a *Vibrio* species control and result showed the LAMP assay 100 times more sensitive than PCR assay with 100% specificity (Xu et al., 2012).

In addition, a multiplex LAMP (mLAMP) assay is developed for simultaneous detection of two or more *Vibrio* species by amplifying their DNA in a single tube. Two or more sets of each of the four LAMP primers with total eight or more primers are used in the same reaction mixture (Biswas et al., 2014). A study on a multiplex LAMP was done for the detection of *V. harveyi, V. anguillarum* and *V. alginolyticus* from Japanese flounder, shark, seabass and shrimp (Yu et al., 2013). Three sets of four species-specific primers were designed and performed in a single reaction. This assay showed 10^2 to 10^3 times more sensitive than the traditional PCR assay. However, in the detection of mLAMP products, the agarose gel electrophoreis was used followed with sequencing or further confirmation of species among these three *Vibrios*. This detection methods are tedious, time consuming, expensive and requires electrophoresis equipments.

Even though LAMP assay offers a rapid, high sensitivity and specificity and user friendly, but it has a complicated primer design especially for new users. As we know, LAMP assay requires more than one set of primers that target six or eight regions within a target DNA (Notomi et al., 2000). Sometimes, the termination step is required to stop the LAMP reaction by heating at 80°C for two to ten minutes (Biswas et al., 2014). Instead of the LAMP assay, the simplest reaction HDA assay can be used since it requires one set of primers and requires reaction buffer and enzyme mix similar to the PCR assay (Vincent et al., 2004). Moreover, this assay can be performed at 60 to 65°C for less than one hour. However in aquaculture field, there is no publication found regarding the detection of *Vibrio* species using HDA assay.

LATERAL FLOW DIPSTICK

Nowadays, many publications in aquaculture field focused on the detection of *Vibrio* species by LAMP assay combined with lateral flow dipstick (LFD). Lateral flow dipstick is used as an alternative detection method to the agarose gel electrophoresis, turbidity, fluorescence and restriction enzyme digestion. LFD detection method is a rapid, user-friendly, and easy to perform, highly sensitive and specific and stable at room temperature (Posthuma-Trumpie et al., 2009). Also, it is able to detect multiple pathogenic bacteria simultaneously in one strip. Thus, a

multiplex LAMP can be developed and detected via lateral flow dipstick. LFD is known as oligochromatography lateral flow or immunochromatographic test (Brandonisio et al., 2002). It is based on the chromatography technique where the reagents were immobilized on the nitrocellulose strip and reactivated using sample/amplification product for the detection of amplicon.

LAMP assay combined with LFD was developed for the detection of *V. alginolyticus* targeting *rpoX* gene (Plaon et al., 2015). The biotin-labeled LAMP product was amplified at 60°C for one hour and hybridized with a fluorescein isothiocyanate (FITC)-labeled probe and result was visualized within five minutes. Result showed that the LAMP-LFD was ten times less sensitive in detection of *V. alginolyticus* with 100% sensitivity and specificity (Plaon et al., 2015). In 2010, Prompamorn was developed the LAMP-LFD for the detection of *V. parahaemolyticus* from shrimp samples. The LAMP amplification was performed at 65°C for 90 mins using primers labeled with biotin. The biotinylated LAMP amplicons were then hybridized with a FITC-labeled probe and detected via LFD for five mins. Result showed the detection limit of the LAMP-LFD was 1.8 X 10^3 CFU g^{-1} which is ten times more sensitive than PCR assay.

Surasilp (2011) was developed LAMP combined with LFD for the detection of *V. vulnificus* targeting RNA polymerase subunit sigma factor S (*rpoS)* gene. The LAMP-LFD was able to detect 1.5 X 10^3 CFU ml^{-1} of *V. vulnificus* from pure culture and also has 100% sensitivity and specificity. Other study showed the LAMP-LFD is successfully developed for the detection of *V. harveyi* from black tiger shrimp, green mussel and bloody clam (Thongkao et al., 2013). The amplification was performed for 60 mins to produce biotinylated-LAMP products and hybridize with FITC-labeled DNA probe and detected via LFD for five to ten mins. Result showed that the LAMP-LFD is ten times more sensitive than PCR assay in the detection of *V. harveyi* from shrimp samples.

The combination of LAMP assay and lateral flow dipstick for the detection of vibriosis offers several advantages such as rapid (30 min to one hour), highly sensitive and specific, equipment free and deliverable to end user. Thus, a multiplex LAMP combine with LFD can be a potential diagnostic test for simultaneously detection of *Vibrio* species from fish, mollusks and other aquatic animals.

CONCLUSION

In conclusion, the isothermal amplification assay especially LAMP is capable for the detection of *Vibrio* species. The simplicity of lateral flow dipstick and LAMP assay offer great potentials for the development of nucleic acid diagnostic devices that could be used to detect vibriosis at point-of-care or in the field.

ACKNOWLEDGEMENT

This work (LAMP development) was supported by the grants provided by Ministry of Higher Institution via Higher Institution Centre of Excellence (HICoE) under Vote No: 6369100.

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