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***Research Article** (Open Access)*

LABORATORY SCALE PRODUCTION OF VIRAL TRANSPORT MEDIUM TO PRESERVE AND TRANSPORT BIOLOGICAL SPECIMENS FOR VIROLOGICAL INVESTIGATION AND VALIDATION USING REAL-TIME POLYMERASE CHAIN REACTION

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ABSTRACT
Viruses are the leading cause of world epidemics and pandemics resulting in the depletion of commercially available inventories of swabs and other consumables creating a huge demand during these periods. The viral transport media was formulated using the CDC protocol in the laboratory using Hanks balanced solution, gelatin, antibiotics and phenol red. The prepared medium is assessed by sterility test, pH meter and RT-PCR. The main aim of the study is to produce VTM and check the viability of the cell using internal endogenous control. It shows that there is neither contamination nor significant changes in the viral transport medium. It suggested that the viral transport medium formulated in the laboratory is indigenous, cost-effective and viable. Hence further research can be carried out to develop this into a commercially available pharmaceutical product.

Keywords: *RT-PCR, VTM, CDC*

INTRODUCTION
The stabilization of samples for the identification of bacteria and viruses, particularly during diagnostic investigations, has been advised for many years using a variety of media solutions. Even though labs have created their own viral transport medium (VTM), commercial preparations are often used. The general VTM formulations consist of a buffered salt solution at a physiological pH, a complex protein and/or amino acid source, and antimicrobial agents**.** In practice, the transport medium can be provided in a 1 dram vial for convenient inoculation or in a standard 15 ml polypropylene centrifuge tube, containing a premeasured 3 ml volume of the medium. The centrifuge tube along with the medium can include glass beads. Typically, four (3 mm) glass beads can be included. This size of glass bead is preferred to prevent them from becoming trapped in the bottom of the centrifuge tube while being vortexed (Racioppi & Brinker,1997)
The isolation and identification of viral agents have greater difficulties because of one outstanding inherent property, namely, their strict intracellular parasitism (Lennette,1972). Routine viral diagnostics include techniques for both indirect and direct detection of viruses. Indirect detection of viruses is performed by serological diagnosis which includes the most common method of viral identification by specific antibodies either by monoclonal antibody or polyclonal serum. Techniques for direct detection of the virus include the detection of viruses include of viral antigens, viruses or viral components by isolation of viruses on viral cell culture and detection of viral nucleic acids also referred to as nucleic acid testing (NAT). Further virus morphological studies can be carried out utilizing electron microscopy (Jerome, 2016). Nucleic acid detection has become more practical and prominent, particularly since the description and development of PCR.
Quality control in the clinical virology laboratory consists of a set of procedures designed to help ensure delivery to the medical staff of laboratory results that are consistent and accurate. These results must be supplied on time. The quality results from the laboratory depending on the quality control (QC) of the testing laboratory. The outcome is significantly influenced by many pre-analytical, analytical and post-analytical factors. According to the Directive on In Vitro Diagnostic (IVD) Medical Devices (98/79/EC) of the European

Union, an IVD must have data proving that it performs as promised and will continue to do so after being transported, stored, and used at its intended location.
Hence it is necessary to validate the prepared viral transport media to check the viability of the specimen and get accurate results for a consistent period of time.

MATERIALS AND METHODS

MATERIALS

Preparation of medium

Modified Hanks Balance Salt Solution Composition (1L)

Table 1: Composition of Hans Balance salt solution (HBSS)

NaCl	6.72 g	115 mM
CaCl2	133 mg	1.2 mM
MgCl2	114 mg	1.2 mM
K2HPO4	418 mg	2.4 mM
HEPES	4.77 g	20 mM
Glucose	1.0 g	10 mM

In 500mL of Hans Balance salt solution (HBSS), 10mL of gelatin was added to the bottle of HBSS and then 2mL of the Gentamicin/Amphotericin B mixture was added resulting in final concentrations of 100µg/mL for Gentamicin and 0.5µg/mL for Amphotericin B. Laboratory appropriate identification (e.g. lot number) was assigned. Then the phenol red was added to the solution. To keep the solution free of contamination was sterilized using a filter membrane with nitrocellulose paper of size 0.45 microns. The lot information was recorded and prepared in a laboratory-controlled notebook.

Validation method

The validation method includes the assessment using sterility check, pH stability and spike test with the help of RT PCR by detecting the presence or absence of RNAase P human control as the method of accelerated stability.

Sterility susceptibility test

Bacteriostatic and Fungi static activity

To check the antimicrobial activity the sterility test was performed against the bacteria and yeast. The bacterial sample that was used was staphylococcus and the yeast sample that was used was *Aspergillus* were employed. It determines the bacteriostatic property and fungal static property of the antimicrobial agents in the VTM.
The sterility test was checked as follows: bacterial or yeast stationary phase cultures was diluted and inoculated into VTM at 103–104 cfu. The VTM was incubated for 3 days at RT (18–20 °c), where 10µl was removed and plated onto blood agar for the bacterial activity and SDA agar for the fungal activity. Growth on plates were compared with the inoculum and reported as no growth (ng), < 10 colonies (+), or > 300 colonies (+ + +) using colony countered. The blood agar plate for bacterial sample and SDA agar for the fungal sample was obtained using a sterile,1 ml pipette, withdraw 1ml of medium as described in the step and apply it to the surface of the blood agar plate and SDA agar plate or equivalent quality controlled plate. The plate was incubated for 48 hours at 37°c ±°2c. Checked daily for growth for 30 days. Recorded results of sterility checked (growth or no growth) and lot specific information in laboratory-controlled documentation.
If bacterial growth or fungal growth has been encountered, took appropriate follow-up actions to remove the bottle of medium from service and dispose of the medium as appropriate.

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Product: Hs_RPP30 Positive Control

Product number: 10006626

Batch number: 0000510597

Expiration date: 2022-FEB-27

Test	Specification	Results
Sequence identity verification (next generation sequencing)	100% sequence coverage with 100% sequence purity	Pass

Storage: Store Hs_RPP30 Positive Control in a sealed container at –20°C.

Verified by: Jacob Petrzelka
Quality release date: 2020-MAR-24

This product has not been clinically validated and is not designed or intended for use in clinical application. The product identified herein is manufactured under IDT ISO certified quality systems in compliance with GMP and in accordance with approved IDT analytical specifications. This certifies that the product has been reviewed and all analytical specifications have been determined to be acceptable. This document was produced electronically and is valid without signature.

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Plate 1: Positive control used in Real-time PCR

Quality control

***Stability assessment*:**

Using in ph meter

Two tubes of VTM were incubated at either 45°C or 4°C (CDC-recommended storage temperature) for 30 days. The stability of VTM was assessed by recording pH for 30 days. One was checked at room temperature and the other was checked by keeping it under refrigeration. The results are recorded daily.

Accelerated Stability test

Real-Time PCR using RNAse P human control

A randomly selected tube of medium from each lot was tested for its support of SARSCOV-2 RT-PCR testing using the researched used authorization (RUA)-authorized true screen Real-Time SARS-COV-2 assay ran on the Applied Biosystem HT7900 platform, the same system as used for clinical testing in our health care network. IDT-covid-19 reference material (recombinant Sindbis virus containing the SARS-COV-2 RNA amplicon target) was spiked into samples of VTM from the lot under study at 2 the assay limit of detection (200 copies/ml) to assess SARS-COV-2 amplification near the assay limit of detection. The reference material used as positive control is shown in Plate 1. The data were recorded for 30 days in the interval of 10 days. All spiked VTM ct values were recorded and visualized in aggregate as a Levey Jennings plot using Excel 2016. The potential contamination of the VTM with the SARS-COV-2 amplicon was also evaluated during testing. This QC assessment was considered to be passed if both the assay internal control(IC) including RNAase p and positive control (SARS-COV-2 amplicon target) demonstrated appropriate levels of amplification with acceptable cycle threshold (Ct) values, reflecting the assay's small inherent native variation in the absence of SARS-COV-2 detection.

The datas was logged for 30 days at 10day intervals. All VTM ct values noted were recorded using Excel 2016 and visualized as Levey Jennings charts. Potential contamination of the VTM with SARS-COV-2 amplicons was also assessed during testing.If the internal control (IC) of the assay, including RNAase p, and the positive control (SARS-COV2 amplicon target) showed appropriate levels of amplification and acceptable cycle thresholds (Ct), then this QC assessment is considered passed, the test of the assay reflects the low variability inherent in the absence of detection of SARS-CoV-2.

Statistical tool

In order to develop Levey–Jennings charts for daily use in the laboratory, the first step was to calculate of the mean and SD of a set of 20 controlled values. This was done in accordance to the WHO laboratory quality management handbook. The number of independent data points (values) in a data set were represented by "n". The mean was calculated and the mean was reduced. Calculating the mean reduces the number of independent data points to n – 1. Dividing by n –1 reduces bias. The values of the mean, as well as the values of + 1, 2 and 3 SD were needed to develop the chart used to plot the daily controlled values. To calculate 2 SDs, multiply the SD by 2 then added and subtract each result from the mean. To calculate 3 SDs, multiply the SD by 3, then added and each result from the mean. The values on the chart were those ran on days 1, 2 and 3 after the chart was made. In that case, the second value was "out of control" because it falls outside of 2 SD. When using only one QC sample, if the value was outside 2 SD, that run is considered "out of control" and the run must be rejected. If it was possible to use only one control, choose one with a value that lies within the normal range of the analyte being tested. When evaluating results, accept all runs where the control lies within +2 SD. Using this system, the correct value will be rejected 4.5% of the time.

RESULTS

Media preparation

The viral transport medium prepared on the basis of CDC formulation was found to be red in color after the addition of phenol red. The prepared viral transport medium is shown in Plate 2.

Plate 2: Formulated viral transport medium

Method validation

Sterility test

Bacteriostatic activity

The sterility test was performed against bacterial specimen *Staphylococcus* in the blood agar medium using direct inoculation and was found to have no bacterial growth. This was observed regularly for 30 days. The absence of bacterial growth indicates the VTM was free of bacterial contamination of VTM. The sterility test against the bacterial specimen in blood agar is shown in Plate 3.

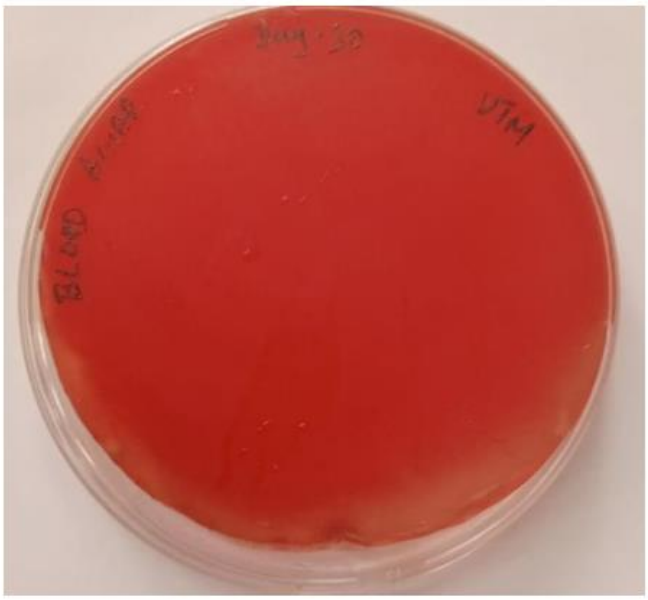

Plate 3: Observation of sterility test against *Staphyloccus* for 30 days

Fungistatic activity

The sterility test was performed against the fungal specimen *Aspergillus* in SDA medium using the direct inoculation method and was found to have no fungal growth. This was observed regularly for 30 days. The absence of fungal growth indicates that the VTM was free of fungal contamination of VTM. The sterility test against fungal specimen is shown in Plate 4.

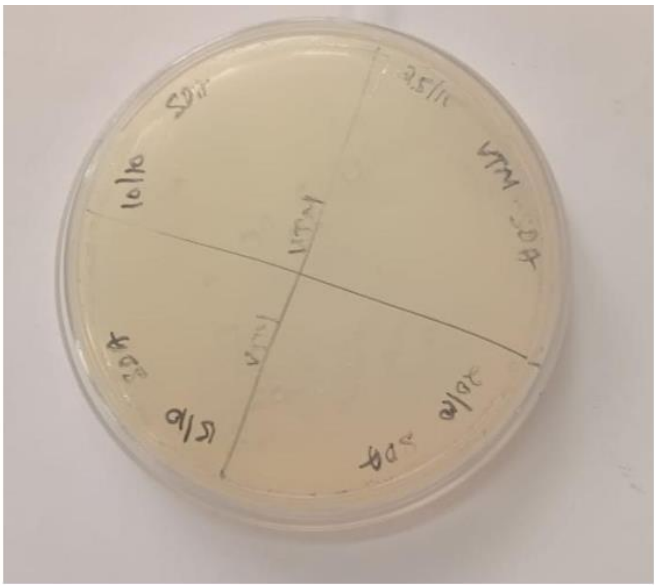

Plate 4: Observation of sterility test against *Aspergillus* for 30 days

Stability test

pH Meter

The two tubes with VTM were used to investigate the pH and were found to have a significantly consistent pH for 30 days both at room temperature and at the refrigeration temperature. One tube was studied at room temperature and the other was studied under refrigeration temperature. The data were observed and recorded for 30 days. The data of VTM under room temperature were represented as a tabular column and expressed using the Levey Jennings chart using excel 2016(Figure 1). The other tube that was observed under the refrigerated temperature was found to be at consistent pH of 7.28 for all 30 days.

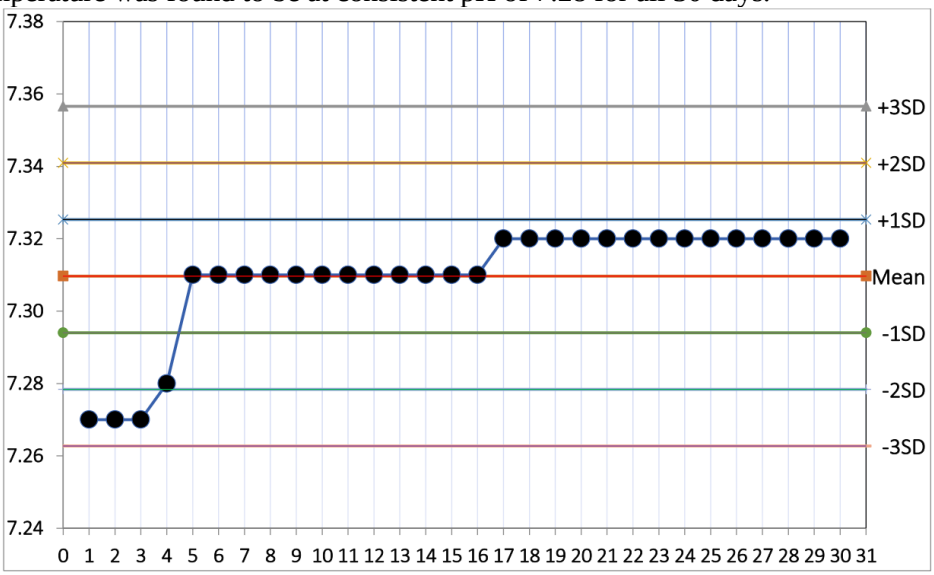

Figure 1: Recorded pH of viral transport medium under room temperature for 30 days

Accelerated stability test spike test using Real-Time PCR

The result produced by Real-time PCR revealed that the viability of the sample is still maintained. There is the presence of the internal endogenous control of RNAse P which has been expressed by the appropriate amplification. The amplification while performing Real-time PCR is shown in figure 2. The recorded ct values are depicted for 30 days in the interval of 10 days in Table 1.

The Ct measured using Real-time PCR through the spike test were expressed using the Levey-Jennings chart with the software excel 2016. The graph shows there was no significant deviation in the Ct value (Figure 3).

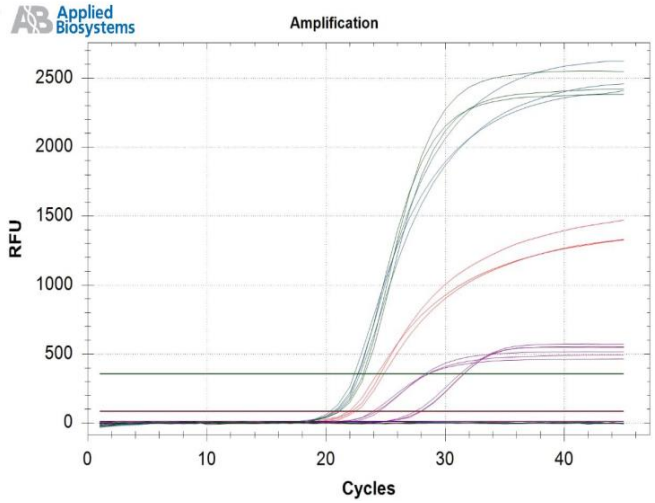

Figure 2: PCR amplification during the validation of viral transport medium

Table 2: Recorded data during PCR amplification for the spike test

Copy Numbers		Ct Value	Days of observation
Theoretical Value	Measured value		
79	72	24.2	1
25.5	30	25.2	10
19.4	16	22.5	20
9.5	10	23.0	30

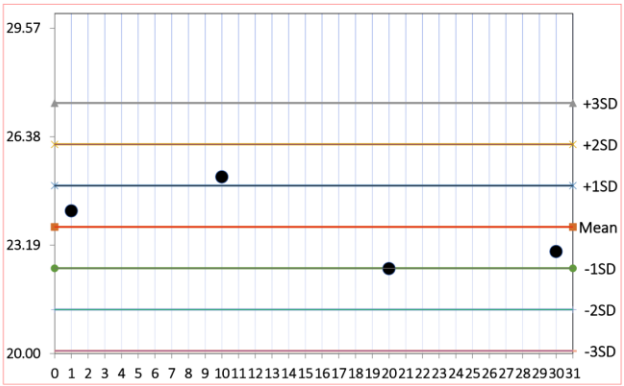

Figure 3: Observation of Ct values for viral transport medium in the course of 30 days under Real Time PCR in the interval of 10 days

DISCUSSION

The phosphate-buffered saline or Hanks BSS (Hanks & Wallace, 1949) has been used as a buffer in a wide variety of transport media from the ancient days. These solutions are considered to approximate the osmotic pressure required by many viruses for optimal stability. Some workers have included organic buffers such as N-2- hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) in their media to provide additional buffering capacity. VTM normally consists of anti-microbial drugs and a protein, like gelatin, in a buffered salt solution. VTM protects specimens from drying, aids in maintaining viral viability, and slows the development of microbial contamination. In labs studying cell biology, phenol red is a typical pH indicator. It is a weak acid that changes the color of something from yellow to fuchsia when the pH is above 8.2. It can be used to visually assess the proper compounding parameters, show whether the solution pH is optimal, and show whether the media (as initially compounded) has subsequently been polluted or exposed to air due to a loose lid. If a laboratory or pharmacy possesses the necessary pH-testing equipment, the use of phenol red may not be necessary; nonetheless, in clinical practice, a visual check may be simpler for each sample and each batch. Phenol red was chosen to be an element of the VAMC's composition because it could be used to identify potential bacterial contamination and change color from pink to yellow in the presence of rising acidity (Petersen *et al*., 2021).

In mammalian cell cultures, gentamicin is the second most frequently utilized antibiotic dietary supplement. Gentamicin has better biological and biochemical qualities than Penicillin and Streptomycin when used singly or in combination. The class of antibiotics known as aminoglycosides includes gentamicin. It is a protein synthesis inhibitor having bactericidal efficacy against mycoplasma, Gram-negative bacteria, and Gram-positive bacteria. Gentamicin has been demonstrated to be pH and temperaturestable37 °C in both acidic and alkaline pH for 15 days without being harmed by the presence of serum. Additionally, after being autoclaved for 15 minutes at 121 °C and 15 lb of pressure, gentamicin was discovered to be totally stable. The standard concentration of gentamicin (50 g/mL) was shown to numerous mammalian cells' shape, proliferation, and metabolism were shown to be unaffected (Hassan & Ahmad, 2020).

Amphotericin B is an antibiotic used as the "gold standard" in the treatment of life-threatening fungal infections. Several molecular mechanisms have been proposed to explain the exceptionally high effectiveness of amphotericin B in combating fungi (Grela *et al*., 2019). Three different types of cultural expression (thioglycolate broth, chocolate agar, and sheep blood agar) were applied at the VAMC to ensure that sterility tests were comprehensive (Petersen *et al*., 2021). Most VTM contain antibiotics to prevent harmful microbe multiplication and are buffered for pH stability (Jensen & Johnson, 1994).

Direct nucleic acid extraction was accomplished using the medium on VTM, UTM, E, and saline swabs.It was discovered that VTM worked with a variety of swab types and, according to accelerated stability testing, could continue to function for at least four months at room temperature (Smith *et al*.,2020). The Ct results for the influenza virus under the tested settings are displayed. Utilization of UTM, VTM, and E was often linked to an amplifiable product. Although Ct results offer some quantitative information, it was not possible to evaluate certain issues, such as the connection between viral load and illness severity (Druce *et al*., 2012). Since the increasing application of molecular-based diagnostic techniques, particularly real-time PCR (qPCR), studies have been conducted to assess the stability of viruses in VTMs, particularly in commercially manufactured products, while stored at a variety of temperatures. While thermal properties have been studied, very little was taken into account of other VTM features or the potential impact of endogenous components (Kirkland & Frost 2020).

CONCLUSION

From the above experiment, it is concluded that the VTM formulated in the laboratory is indigenous, cost-effective and viable. All reagents necessary for the CDC formulation are commonly used for cell culture and were already available in our research division and available to order. The quality of the viral transport medium is maintained even at room temperature. It is able to sustain the viability of the biological specimen. The sterility test shows that the viral transport medium is free of contamination. The use of gelatin instead of fetal

bovine is a cost-effective method and an excellent alternative to full fill the purpose. Thus the viral transport media prepared is suitable to collect, transport and store biological specimens with viruses for molecular diagnostics.

The viruses are the leading cause of world epidemics and pandemics resulting in the depletion of commercially accessible inventories of swabs and other consumables creating a huge demand during these periods. Concerning the recommendation our strategy was lab-based neither viral samples nor actual patients were engaged. Hence further research can be carried out using viruses in this formulated viral transport medium to develop this into a commercially available pharmaceutical product.

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