Rapid detection of φX-174 virus based on synchronous fluorescence of tryptophan

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Received: 6 September 2022 / Revised: 9 November 2022 / Accepted: 10 November 2022 / Published online: 28 November 2022
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Abstract
The development of rapid methods for the detection of virus particles based on their intrinsic fluorescence (the native auto-fluorescence that originates from the non-labeled analyte) is challenging. Pure viruses may be detected in filtered solutions, based on the strong fluorescence of the amino acid tryptophan (Trp) in their proteins. Nevertheless, Trp also exists in high quantities in the hosts and host cultivation media. In this work, we developed a new method for the detection of the naked φX-174 virus. We show that a separation of φX-174 from its Escherichia coli host (grown on the standard cultivation medium nutrient agar) by simple extraction and filtration is not sufficient for its detection based on the intrinsic fluorescence since ~70% of the Trp fluorescence is derived from impurities. We formulate a new cultivation medium with a very low Trp concentration. We apply synchronous fluorescence measurements to show that no Trp fluorescence is detected in the extract solution upon incubation of this medium substrate with ammonium acetate extraction buffer. Finally, we apply synchronous fluorescence to detect φX-174 based on the spectral fingerprint of its native Trp content. Such a method is more rapid than usual traditional separation and detection methods which can take several hours and does not require any addition of labeling agents such as fluorescent dyes or antibodies for the detection. As other virus species contain Trp as one of the amino acids presents in their proteins, this method has the potential to apply to the detection of other viral species.

Keywords Detection · φX-174 · Tryptophan · Synchronous fluorescence · Cultivation media

Introduction
Over the years, different anti-bacterial agents, such as antibiotics and various peptide-based drugs, were successfully developed against bacterial pathogens [1]. However, in the case of viral pathogens, many infectious diseases have crossed the barrier, the equilibrium between the virus and the human host, causing a high disease burden and mortality [2]. In recent years, the global pandemic (caused by the SARS-CoV-2 virus) has spread worldwide, causing millions of deaths and severe illnesses [3]. Detecting the viral absence or presence enables quick isolation of the carrier, to prevent them from infecting additional people. Therefore, it is vital to develop sensitive and rapid viral detection methods.

Classical detection methods utilize bacterial or mammalian cells as hosts for the viruses. The cell lysis that is caused by the viruses forms discrete visible zones (plaques) that can be detected by the eye or microscopic observation [4]. Purification of viruses from their host cell debris and other contaminants is typically done by density gradient centrifugation [5], ultrafiltration [6], or different chromatographic methods [7, 8]. The small sizes of viruses make them invisible to light microscopic observation and can be done by transmission electron microscopy (TEM) [9] or by adding fluorescent labeling agents under advanced microscopic instrumentation [10]. The determination of viral structures is extensively investigated by X-ray crystallography, NMR, and...
cryo-TEM [11, 12]. A high purification degree of viruses is essential for their structural and fundamental studies, so it may be time-consuming. For instance, purification of adenovirus-associated virus by density gradient centrifugation is taking 112–120 h [5]. Therefore, such methods may not be feasible when rapid detection is required. The identification of virus can be done by biosensors without an extensive purification process [13–17]. In most cases, such biosensors are based on immunoassays in which unique antibodies can target specific viruses. The bound antibodies can be identified by simple gel electrophoresis or immunofluorescence methods [18].

Another precise identification method is next-generation sequencing (NGS) which can sequence the whole genome of the virus [19]. Nevertheless, whole genome sequencing is still relatively expensive for large-scale applications. A cheaper method for detection that is extensively used in recent years is polymerase chain reaction (PCR) [11, 12]. This method can detect viruses by the amplification of specific sequences in the viral genome. An enhanced sensitivity was achieved by the development of loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) [20–24]. However, PCR needs few hours for preparation of viral RNA and can be executed by specialists only [17].

Different spectroscopic methods were reported to identify isolated viruses. Raman spectroscopy was able to identify viruses [25] and also help in determining and characterizing their structure [26–28]. The amino acids tryptophan, tyrosine, and phenylalanine have an intrinsic fluorescence and can be used to detect isolated viruses [29]. Intrinsic fluorescence can also be measured from nucleic acids; however, the intensity is relatively low and, in many cases, may not be sufficient to be detected [30]. Nevertheless, the fluorescence intensity of nucleic acids can be detected by the utilization of labeling agents such as SYBR dyes, which can significantly amplify it [31].

Several scanning methods are commonly available in commercial fluorimeters. The scanning method which is most extensively used is the emission scan in which the excitation is performed at a single wavelength and a wide range of emission wavelengths is collected. A more advanced scanning method is synchronous fluorescence (SF) [32, 33]. In this scanning method, both the excitation and emission wavelengths are scanned while keeping a constant wavelength interval (Δλ) between them. This is equivalent to scanning along a line of slope 1, in the 2D excitation–emission map. When the wavelength interval Δλ between the excitation and emission wavelength is chosen properly, the resulting spectrum will show one or several features that are more resolvable than those in the conventional fluorescence emission scan. The maximum fluorescence intensity for a particular component occurs when Δλ corresponds to the difference between the wavelengths of the excitation and emission maxima for that component [34, 35]. SF spectra are often narrower than the emission spectra, and when mixtures are analyzed, selecting a proper Δλ may reveal more peaks, and overlapping of multiple spectral components can be minimized [36, 37]. Intrinsic SF of Trp has been extensively used for many analytical applications. Among them are studying molecular mechanisms in bacteria [38] and in proteins [39], early detection of carcinoma in mice [40], and monitoring potent anticancer effects in glioblastoma cells [41]. While SF methods have been demonstrated as a rapid tool for bacterial classification and identification [42–45], and even distinguishing between live and dead bacteria [46], to our best knowledge, no detection of viruses by intrinsic SF has been reported.

Fluorescence spectroscopy has great potential for rapid detection of pure viruses based on the intrinsic fluorescence of their amino acids. However, it is not compatible with matrices with many biological compounds that also consist of the same amino acids. In this work, we report a novel medium composition for the cultivation of the bacterial host of the virus φX-174, whose fluorescence contribution does not have any significant overlap with Trp. Also, we demonstrate that this medium enables the detection of the spectral fingerprint of φX-174. This was achieved by a simple filtration followed by SF. φX-174 is a naked virus type which is considered as a model for structural biology [47] and exhibit structural similarity for several pathogenic viruses such as poliovirus or parvovirus [48].

Materials and methods

Replication of the virus φX-174

To replicate the virus φX-174, we used the “double-layer” method [49]. Briefly, in this method, a host bacterium (in high concentration) is grown in a solid medium together with infectious phage particles. After the phage replicates itself in the bacterium and lyses the bacterium cells, a visible clear zone (plaque) is formed. In our case, to Escherichia coli CN13 (ATCC 700609, a nalidixic acid-resistant strain), a φX-174 (DSM 4497, Germany) was added and grown on nutrient agar (NA) composed of nutrient broth (Difco, USA) with 0.7% agar (Difco, USA). After overnight incubation (37 °C), viral plaques were formed.

Purification of the virus φX-174 from whole E. coli cells

For separation of viral particles from the whole E. coli cells, 5 ml ammonium acetate buffer (ammonium acetate, Riedel–de Haen, Germany, pH = 7, 0.1 M) was added to the “double-layer” agar (Petri dish of 100 mm diameter).
The buffer was floated on the agar for 10 min and then was pumped out by syringe and filtered by PVDF sterile syringe filter (0.22 μm, Millipore, Ireland). To estimate the contribution of lysed host bacterium cells and diffused molecules from NA on the SF measurements, the 0.22-μm filtrate was filtered again by 0.02 μm alumina membrane (Anodisc filter, Whatman, Germany) and vacuum pump. The alumina membrane was kept in dark at 4 °C (for one overnight only) before tested for SF measurements the day after.

Preparation of LTM

The LTM consisted of glucose, 10 g/L (Merck, Germany); KH$_2$PO$_4$, 5 g/L (Carlo Erba, Italy); K$_2$HPO$_4$·3H$_2$O, 5 g/L (Merck, Germany); (NH$_4$)$_2$HPO$_4$, 3 g/L (Merck, Germany); Na$_2$HPO$_4$, 4.6 g/L (Fluka, Switzerland); MgSO$_4$·7H$_2$O, 0.6 g/L (Merck, Germany); yeast extract, 0.5 g/L (Difco, USA); and Agar, 7 g/L (Difco, USA).

SF measurements

All SF measurements were performed using a Fluorolog 3 fluorimeter (Horiba) with excitation and emission slit bands of 7 nm at Δλ = 60 nm.

Theoretical calculation of Trp units in φX-174

The theoretical number of Trp units in φX-174 was calculated based on the sequences of the main proteins in φx-17, capsid protein F, major spike protein G, minor spike protein H, and DNA-binding protein J, which are deposited in the protein data bank (PDB (www.rcsb.org/pdb)).

Results and discussion

Tryptophan residues in the cultivation media limit the detection of φX-174 by synchronous fluorescence

A frequently used method for replication of φX-174 in Petri dishes is called the double agar layer method [49]. In this method, the virus replicates within E. coli that is cultivated on top of a layer of growth medium such as nutrient agar (NA). Whole bacterial cells can be easily separated from φX-174 by simple filtration or centrifugation. However, peptides or proteins, which contain Trp, may also originate from lysed bacterial cells or diffuse from the lower layer of the cultivation media, making the purification of φX-174 more challenging. In our previous work [50], we showed that the concentration of Trp can be directly determined by applying a single SF scan with optimal conditions of Δλ = 60 nm. Based on our previous studies, in these conditions, it is possible to differentiate between the emission of Trp and the other two fluorescence amino acids phenylalanine (Phe) and tyrosine (Tyr). The peak that originates from Phe has a maximum at ~ 310 nm and does not have a significant overlapping with Tyr and Trp that have a maximal emission at ~ 340 and 350 nm, respectively. Although the spectra of Tyr and Trp partially overlap, Tyr has no significant contribution at 350 nm, making it an optimal wavelength for Trp quantification [50]. We wished to utilize SF to explore whether lysed cells and diffused molecules from the substrate limit the detection of φX-174 based on its Trp content. φX-174 plaques were replicated on an E. coli layer on top of NA agar. The φX-174 was extracted by ammonium acetate buffer for 10 min. The supernatant was removed and filtrated through a 0.22-μm filter and again by 0.02 μm. The same procedure was repeated for Petri dishes without φX-174. To evaluate the Trp concentration of the filtrates, SF measurements were performed on an alumina filter (Δλ = 60 nm, λ Em (280–450 nm) (Fig. 1). The spectral shapes in all measurements were consisted of a peak with a maximum at ~ 350 nm that has the exact spectral shape of Trp without any significant contribution from Tyr [50]. Based on this, we suggest that at Δλ = 60 nm, the fluorescence contribution of Tyr was below the noise level. The ratio between the maximal intensities of the filtrates (λ Em = 345 nm) without and with φX-174 was 68.8%. These results show that the high fluorescence contribution of the NA or lysed bacterial cells highly overlaps with the fluorescence that originates from φX-174. This overlap limits the ability to make a reliable detection based the Trp content of the virus.
Preparation of a cultivation medium with low amino acid content

To decrease the Trp fluorescence contribution that derives from the bacterial cultivation medium, we prepared a new medium that consists of less Trp than in NA. The main ingredients of the low Trp medium (LTM) were agar, glucose, a salt mixture, and yeast extract (for more details, see the experimental section). *E. coli* was successfully cultivated on LTM to form a uniform layer.

Next, we wished to estimate the spectral interference of each group of ingredients and the mixture of all ingredients on spectral overlap with Trp. Media with agar, agar + glucose, agar + salts, agar + yeast extracts, and agar + glucose + salts + yeast extracts (YE) were prepared. Ammonium acetate buffer was added to the top of the substrates and incubated for 10 min. The buffer was removed and filtrated. SF spectra of the filtrates (Δλ = 60 nm, λ_em = 310–370 nm) were measured (Fig. 2). The filtrates of the agar and agar + glucose extraction did not have a significant fluorescence contribution. A small fluorescence contribution of 0.1 counts per second (CPS) was obtained from the agar + salt extract. However, the shape of the peak was not similar to the fluorescence peak of Trp. A peak with a significant intensity of 0.6 CPS was obtained for the YE extract. The intensity of this peak is ~5 times less than that obtained when using NA media (Fig. 1). Interestingly, when all ingredients were added to the substrate, no significant fluorescence contribution was observed at the emission wavelength range of Trp (310–370 nm). Apparently, the presence of sugars and salts increases the viscosity of the substrate and, in this way, significantly decreases the diffusion rate of Trp (and other components) from the cultivation media to the ammonium acetate extraction buffer.

Detection of φX-174 based on its Trp fluorescence fingerprint

Using the LTM reduced the extraction of proteins from the substrate to the ammonium acetate buffer. However, proteins and other fluorescent materials may be secreted from the *E. coli* cells or be released to the buffer by dead or lysed cells. We wished to assess whether such materials are extracted into the ammonium acetate buffer and whether their fluorescence overlaps with the spectral fingerprint of Trp. For this reason, *E. coli* was cultivated on the LTM with or without the addition of φX-174. Ammonium acetate buffer was added to the top of the LTM for 10 min. Then, the buffer was removed and filtrated through a 0.22 µm filter, and the SF of the filtrates was measured (Fig. 3). A fluorescence contribution between 330 and 370 nm was obtained from the sample without φX-174. However, its spectral shape was linear and clearly did not originate from Trp, while the samples with φX-174 showed the spectral fingerprint of Trp. Cells that φX-174 lysed were spilling their NAD+ and NADH pools into the external media. The fluorescence contribution derives from residues of NAD+ and NADH molecules may overlap if the concentrations are high enough [51]. The obtained result shows that when using the LTM substrate (instead of the conventional NA), it is possible to use SF to detect the Trp fluorescence contribution which originates only from φX-174 and not from the host cultivation medium.
To evaluate the Trp concentration, the SF spectrum of the sample without φX-174 was subtracted from the sample with it. Calibration curves of concentration versus the intensity at $\lambda_{em} = 350$ nm were prepared by measuring the SF spectra of Trp. The concentration of Trp in φX-174 was determined to be $4.4 \pm 0.3$ nM.

A plaque assay was conducted to evaluate the number of virus copies in the extraction buffer (for a more detailed explanation, see “Materials and methods”). Based on the plaque assay, the concentration of Trp per virus was evaluated to be $4.4$ zeptomol/PFU (that is equivalent to $2650$ Trp units).

To compare this experimental result with the theoretical number of Trp copies, we performed a bioinformatic analysis based on the deposited protein sequences that are deposited in the protein data bank. In this analysis, we summarized the total number of Trp units in all of the copies of the main proteins in φX-174: capsid protein F, major spike protein G, minor spike protein H, and DNA-binding protein J. The calculated number of Trp units was $456$, about $5$ times lesser than the one obtained in our experiments. We suggest that this difference may derive from the Forster energy transfer (FET) between tyrosine and Trp which are closely associated in the virus particle. This FET may enhance the fluorescence intensity of Trp [52–54].

A model for the method of the φX-174 purification and detection

Based on the presented results, we propose a mechanism that explains the basic principles of the replication, purification, and detection of φX-174 based on the utilization of LTM and the application of SF (Fig. 4).

Conclusions

In this work, we present a new method for fast screening of the virus φX-174 grown on bacterial substrates. φX-174 is a naked virus type that is considered a model virus whose
structure is similar to several pathogenic viral species. We developed a formulation for bacterial cultivation medium which lowers the concentration of Trp in comparison to NA, which is the frequently used cultivation medium. We show that by using this substrate as a platform for the cultivation of E. coli and replication of the virus φX-174, it is possible to detect φX-174 based on its intrinsic fluorescence which originates from its Trp content. We demonstrate for the first time the possibility of using intrinsic fluorescence to detect a virus. The concept of coupling intrinsic fluorescence-based methods to the decrease the impurities in the source may be implemented for rapid detecting of pathogenic virus. This separation and detection method has several advantages upon other existing one. First, it is simple and rapid (only a few minutes) and does not require expensive instruments nor time-consuming stages. Second, the identification is based on the native fluorescence of the virus, without the need to label it with dyes or antibodies. This work was conducted only on a single virus species, and an interfering test, when other viruses are present, is needed. However, it holds the potential to become a general method that is compatible for the detection of other virus types, since all virus species consist of Trp in different quantities.

Acknowledgements We thank Prof. Noam Adir for his scientific consultation in the writing of the paper.

Author contribution YF and YS conceived the idea. YF and YS designed the experiments. YF and YS performed the main experiments. YF and YS wrote the paper. IS and RA supervised the entire research project and provided funding.

Funding This study has been supported by an internal fund (Micro Grants for the Technion Recycling Initiative, Technion, Israel). Yaniv Shlosberg is supported by the National Institutes of Health R35 Maximizing Investigators’ Research Award (MIRA, R35GM142920).

Declarations

Conflict of interest The authors declare no competing interests.

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