Pichia kudriavzevii yeast exposure increases the asthmatic behavior of alveolar epithelial cells *in vitro*

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SUMMARY

Asthma is a respiratory disorder that is characterized by the obstruction of the airway due to mucus overproduction and inflammation. More than 334 million people worldwide are affected by asthma. *Pichia kudriavzevii* is a spoilage yeast that is abundant in the environment and therefore a proficient pathogen. In this study, we observed the asthmatic effects of *P. kudriavzevii* on alveolar epithelial cells through a trypan blue assay (viability), TUNEL assay (apoptosis), PAS stain (mucin presence), and an ELISA immunoassay (IL-6 hypersecretion). Results from the assays displayed a direct correlation between infection duration and asthmatic characteristics. Thus, the *P. kudriavzevii* yeast may be a common cause of asthma-related symptoms and is a worthy cause of further study.

INTRODUCTION

Asthma is a multicellular respiratory disorder that affects more than 334 million people worldwide (1). It is mainly characterized by the obstruction of the respiratory tract due to increased mucus production by the airway's epithelial cells and inflammation of its smooth muscle. It is currently believed that inhaled environmental stimuli trigger the molecular and cellular pathways that drive the clinical manifestations of asthma and other respiratory diseases (2, 3).

The major molecular and cellular indicators of asthma include elevated cytokine production, overproduction of mucus, and increased apoptosis of airway epithelial cells. Environmental stimuli trigger the production of cytokines, such as IL-25 and IL-33. These cytokines promote the production of other cytokines, such as IL-6 (4). IL-6 is known to contribute to the deterioration of the lungs and their function. A 2011 study suggested that a polymorphism in peripheral blood mononuclear cells (PBMC) was largely responsible for the magnification of several asthma-related symptoms, primarily through the induction of cytokine overproduction (5). These altered cytokine levels are also known to contribute to decreased viability of airway epithelial cells. A study identified the exfoliation of airway epithelial cells, as well as the thickening of the basement membrane of the bronchi, as crucial indicators of asthma in patients (6).

In addition to the increased production of cytokines, environmental stimuli trigger the increased production of mucus. Mucus is a viscous solution that is produced by the mucous membranes lining the epithelial surfaces of organs, such as the surfaces of airways in the lungs. Mucus is composed of antiseptic enzymes and glycoproteins. In

asthma, repeated exposure to environmental stimuli promotes secretory cell hyperplasia, the overabundance of mucin-secreting goblet cells in the epithelial layer of the airway, which leads to mucus overproduction (7). Although mucus overproduction in asthma originates in the large airways (structures such as the primary bronchi that are >2mm in diameter), it also extends to the small airways (central and peripheral airways <2mm, such as the bronchioles and alveoli) as the blockages break off, continue travelling down the respiratory tract, and impair airflow and oxygen exchange in the smaller structures of the lungs (8). In addition, it is not uncommon for airway epithelial cells to apoptose as a result of asthma-related eosinophil overproduction. In fact, it is one of the crucial indicators of the disease. A study identified the exfoliation (a form of apoptosis) of airway epithelial cells, as well as the thickening of the basement membrane of the bronchi, in asthmatic patients as crucial indicators of the disease (6).

Some cytokines, including IL-25 and IL-33, are released at the moment that environmental stimuli interact with airway epithelial cells. These cytokines go on to produce other cytokines such as the one we tested in this study: IL-6 (4). IL-6 is an inflammatory marker with a key role in the development of asthma due to its fundamental contribution to the deterioration of the lungs and their function. IL-6 is present in elevated quantities within the bronchoalveolar fluid (BALF) in the lungs of asthmatic patients and upon secretion by alveolar epithelial cells, it augments asthma symptoms by inducing inflammation in the smooth muscle cells of the airway (5). Airway epithelial cell viability in asthma is also often decreased due to the elevated cytokine levels and overall respiratory system dysfunctionality. In fact, a 2011 study suggested that a studied polymorphism in peripheral blood mononuclear cells (PBMC) was largely responsible for the magnification of several asthma-related symptoms, primarily through the induction of cytokine overproduction (5). This further supports the importance of cell viability in the epithelium of asthmatic subjects and its ability to potentially indicate the occurrence of the disease within such asthma patients.

In this study, we will test the effects of *Pichia kudriavzevii* on the cell viability, apoptosis, cytokine production (IL-6), and mucus production of A549 alveolar epithelial cells. *P. kudriavzevii* is a low pH spoilage yeast and is an anamorph of *Candida krusei*. *P. kudriavzevii* can be found in the natural and commercial environments, including in fruits and their many commercial derivatives (9). Thus, this yeast can be considered easily acquirable through exposure to its

intermediate hosts. In addition, *Candida albicans,* a closely related species of *P. kudriavzevii*, has been thoroughly studied, and many studies concluded that it is a primary cause of asthma-related symptoms in asthma patients (10). Data from multiple studies have continuously suggested the opportunistic pathogenic potential of *C. albicans* in hosts with a compromised immune system. These data have especially emphasized the yeast's potential role in the development of asthma and the augmentation of preexisting asthma symptoms in affected patients (11). A foundational study on *C. albicans* also observed yeast-initiated wheal and flare reactions in approximately 58% of the tested asthma patients, thus strengthening the hypothesis that *C. albicans,* and possibly other related species, may considerably contribute to the pathophysiology of asthma in some patients (12).

Although the properties of *P. kudriavzevii* have not yet been tested on any cell lines in order to evaluate its potential effects, an ongoing *in vivo* study heavily suggests there is a connection between the yeast and asthma (13, 14). As such, the possibility exists that *P. kudriavzevii* exhibits similar properties as its anamorph *C. albicans* and is therefore responsible for the asthma-related symptoms in its affected subjects. This study thus aims to understand the molecular asthmatic effects of *P. kudriavzevii* yeast secretions on A549 alveolar epithelial cell behavior, and in doing so increase the understanding of environmental allergens and their effects on the pathogenesis of asthma. Additionally, this study aims to recognize the asthma-related effects of *P. kudriavzevii* on the airway in order to build the foundation for future research on allergen-targeted asthma treatments.

RESULTS

Alveolar epithelial cells exposed to *P. kudriavzevii* exhibit increased cell death
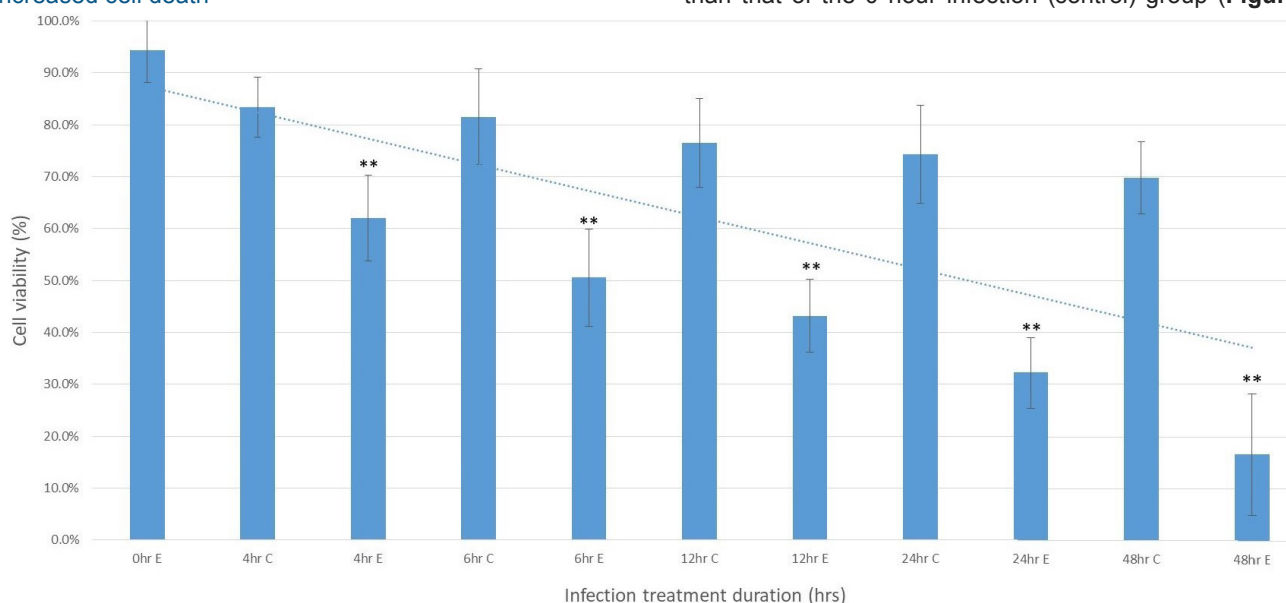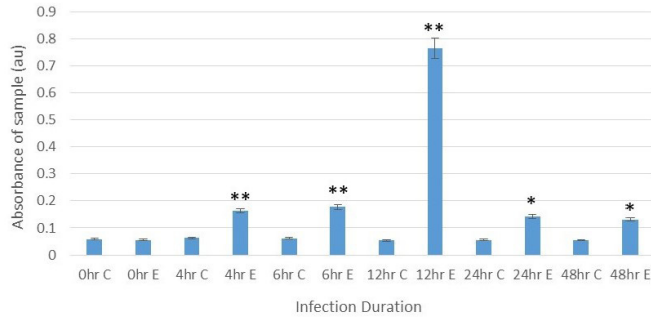
Previous literature suggests a strong correlation between increased exposure to asthmatic agents and subsequent decreased cell viability. Thus, decreased cell viability was a predicted effect of *P. kudriavzevii* infection of A549 alveolar epithelial cells. To measure the cell viability prior to and after cell exposure to the yeast, a trypan blue assay was performed. Non-viable cells were stained blue due to trypan blue's penetration of the damaged cell membranes of dead cells and were counted using the ImageJ software. Treated (experimental) and untreated (control) groups of alveolar epithelial cells were tested via the trypan blue assay at 0, 4, 6, 12, 24, and 48 hours of incubation. All infection treatment durations displayed statistically significant differences (**Figure 1**). We also saw an indirect correlation between cell viability and increased duration of infection treatment. Thus, the data from the trypan blue assay strongly supported the hypothesized, progressively worsening effects of *P. kudriavzevii* treatment on alveolar epithelial cell viability.

Alveolar epithelial cells exposed to *P. kudriavzevii* also exhibited increased levels of IL-6

Another major indicator of elevated asthma-related cellular events is IL-6 hypersecretion. Previous studies have established a strong correlation between elevated IL-6 concentrations and increased asthma progression (17). Thus, we tested IL-6 concentrations both prior to and after *P. kudriavzevii* infection, in order to draw conclusions regarding the effects of the yeast on asthma-related events occurring in these cells. Treated (experimental) and untreated (control) groups of alveolar epithelial cells were tested via the ELISA IL-6 immunoassay at 0, 4, 6, 12, 24, and 48 hours of incubation. Every variation of the experimental infection duration was found to have significantly higher levels of IL-6 than that of the 0 hour infection (control) group (**Figure 2**).

Figure 1: Cell viability as measured by trypan blue assay. Each bar represents the average cell viability within the 10 samples assayed per duration time. The letters E and C adjacent to the infection durations in the bottom legend indicate experimental infected cells and control non-infected cells, respectively. Error bars indicate 95% confidence interval, * $p < 0.05$, ** $p < 0.001$.

Figure 2: IL-6 concentrations in cell samples measured by ELISA. Average absorbance readings from each treatment duration were plotted against the IL-6 standard curve to obtain an approximate concentration of IL-6 present in the sample. E and C indicate experimental infected cells and control non-infected cells, respectively. Error bars indicate 95% confidence interval, *$p<0.05$, **$p < 0.001$.

Thus, a valid conclusion is that in all the experimental groups, the *P. kudriavzevii* infection triggered the hypersecretion of IL-6, a fundamental asthmatic indicator, by the alveolar epithelial cells. However, the data also displayed results that were not statistically significant in the IL-6 concentration differences between the infection groups of 4, 6, 12, 24, and 48 hours, thus suggesting that the production of IL-6 by A549 alveolar epithelial cells peaks after only 4 hours of *Pichia kudriavzevii* stimulation (**Figure 2**).

Alveolar epithelial cells exposed to *P. kudriavzevii* exhibited elevated levels of polysaccharides, the primary precursor to mucus overproduction in organisms

Increased levels of polysaccharides, quantified with the PAS staining assay, is a critical indicator of asthma-related cellular symptoms and is an excellent marker of future mucus hypersecretion. As such, we performed a PAS staining assay to stain polysaccharide contents in the cell samples, and thus measured quantities of polysaccharides in A549 cells both prior to and following *P. kudriavzevii* exposure. Similar to the results from the trypan blue assay, results from the PAS staining assay showed sequentially higher concentrations of polysaccharides in the cell samples as the duration of the infection treatments increased (**Figure 3**). The control, as opposed to the experimental samples, remained constant in its low polysaccharide levels throughout the 48 hours in culture without any exposure to the *P. kudriavzevii* yeast. Thus, once again, a direct correlation between increased asthmatic indicators (in this case mucin overproduction) and increased duration of infection treatment was established.

Alveolar epithelial cells exposed to *P. kurdiavzevii* yeast exhibited increased levels of apoptosis in most cases

Cell apoptosis is a fundamental indicator of ongoing asthma-related events at the cellular level. Quantification of apoptotic cells in both the control and experimental samples therefore gives insight into the pathogenesis of the disease and its manifestations at the cellular level. As such, the TUNEL assay was conducted to identify cells undergoing apoptosis by staining them a dark shade of brown, which could easily be identified and quantified using the ImageJ software. The dark-stained apoptotic cells significantly increased in the infection treatment groups of greater durations, compared to those of shorter durations and the 0 hr (control) group (**Figure 4**) (student's t-test, $p<0.05$). Although only infection duration groups of up to 6 hours were able to be tested via this assay, we established a direct correlation between asthmatic cellular indicators (in this case apoptosis) and duration of infection treatments.

DISCUSSION

Currently, over 300 million patients worldwide reportedly suffer from asthma, and that number increases in developing countries and regions of the world where medical service is largely inaccessible (1). Successful identification of *Pichia kudriavzevii* as a biological asthmatic agent at the cellular level allows for further identification of inorganic asthmatic agents in the biosphere, such as its intermediate hosts in the natural and commercial environments. Analysis of the molecular composition of the yeast secretions responsible for the asthmatic cell behavior can lead to the identification of crucial, molecular asthmatic agents that can be targeted in novel asthma treatments and thus aid in their development. Our experiments yielded results that supported the proposed alternative hypothesis that *P. kudriavzevii* plays a role in the initiation of asthma-related symptoms and events at the cellular level. In all four of the conducted assays, the infection treatments of sequential durations all exhibited significant increases in asthmatic indicators, when compared to the control group. Furthermore, results from many of the conducted assays showed a strong direct correlation between infection duration and asthmatic cellular behavior. More specifically, we concluded that alveolar epithelial cell exposure to *P. kudriavzevii* resulted in decreased cell viability for all infection durations, increased IL-6 secretion in all infection durations, elevated polysaccharide contents in all infection durations, and significantly higher levels of apoptotic cells in the 4 hour and 6 hour infection durations. Furthermore, the effects seen in cell viability, mucus overproduction, and
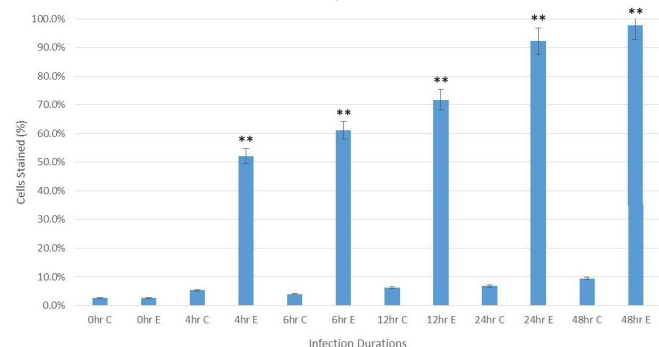
Figure 3: Mucus production by A549 alveolar epithelial cells as measured by the PAS staining assay. The bar graph displays the average results from the 10 samples in each treatment group. Error bars indicate 95% confidence interval, *$p<0.05$, **$p < 0.001$.
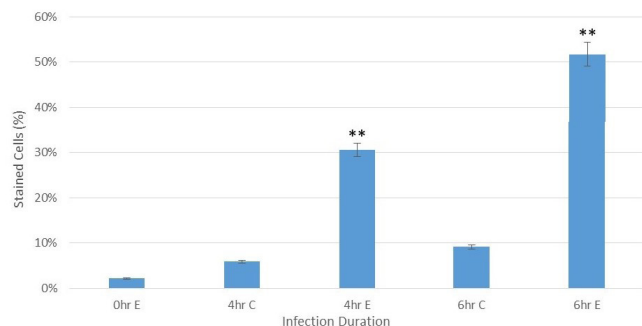
Figure 4: Apoptosis in A549 alveolar epithelial cell samples as measured by the TUNEL assay. Each bar represents the average number of apoptotic cells within the 10 samples assayed per treatment duration. Error bars indicate 95% confidence interval, **$p < 0.001$.

apoptotic cell content were all magnified as the duration of the *P. kudriavzevii* exposure increased. In contrast, IL-6 concentrations were elevated in all experimental groups, but failed to show a direct correlation with the duration of the infection, suggesting that IL-6 hypersecretion is a fundamental asthmatic indicator that is elevated after contact with the yeast yet is independent of the duration of this contact. To further support the conclusion that *P. kudriavzevii* was the initiator of these various asthmatic symptoms, uninfected control cells were grown simultaneously and assayed at the established experimental intervals along with the experimental infected cells (**Figures 1-4**). The untreated (control) groups at the various time intervals exhibited no significant asthma-related symptoms, thus suggesting that the *P. kudriavzevii* infection was the sole initiator of the asthmatic behavior in the alveolar epithelial cells.

The TUNEL assay for apoptosis was successfully conducted in infection duration groups of 0, 4, and 6 hours, and the data collected provided evidence towards a direct correlation between asthmatic cellular indicators (in this case apoptosis) and duration of infection treatment. Interestingly, we could not assess apoptosis in the remaining infection durations (12, 24, and 48 hours), due to the cells' excessive detachment from the coverslips. This suggests that the *P. kudriavzvevii* yeast may have induced cell lifting at an unprecedented level in the samples with longer infection durations. In response to this observation, two new groups in-between the attaching 6-hour group and detaching 12-hour group were created to observe cell adherence both before and after exposure to *P. kudriavzevii*. The two groups had exposure durations of 8 and 10 hours, respectively. We observed significant cell detachment in the 8 hour and 10 hour groups in the post-infection condition compared to the pre-infection condition. These findings provided additional evidence for the hypothesized negative asthmatic cellular effects of the *P. kudriavzevii* yeast on alveolar epithelial cells. Improper cell attachment is a sign of upcoming apoptosis and airway remodeling, both fundamental steps in the asthmatic pathway. Induced cell lifting could indicate worsened cell function, another important characteristic of asthma. The

relationships established by these data also support the direct correlation between asthmatic cellular indicators (in this case cell detachment) and duration of the infection treatment.

The next step in the expansion of this research would be to include an additional model cell line or other model organisms to further expand the perspective of the cellular asthmatic effects of *P. kudriavzevii* yeast. This expansion could give additional insight into the true asthmatic physiological effects of the yeast secretions on the two main types of cells involved in asthmatic pathways within the respiratory tract (alveolar epithelial cells and smooth muscle cells). By testing the inflammatory responses of this cell type when exposed to the yeast infection treatment, a greater understanding of the pathogenesis of asthma can be achieved. Thus, a subsequent step in the broader analysis of these asthma-related symptoms would be to conduct similar experimentation using tissue from the respiratory tract as the model of the study. By doing this, we could identify many properties that would not be otherwise involved in the individual cell line models. These properties could play a major role in the reaction to the yeast secretions and thus potentially dictate a different result with a much wider variety of implications that could augment the results exhibited in this study by the singular A549 cell line. Testing the short-term asthmatic effects of these *P. kudriavzevii* yeast secretions at an organismal level would greatly contribute to the understanding of its molecular, cellular, and physical effects on a living, functioning organism. A worthy potential model organism, and a relatively new addition to the known model organisms for asthma research, the *Drosophila melanogaster* mutant for asthma, has been used in recent studies involving the effects of gene therapy on asthma-related behavior in the cells of the airway (18).

As such, by gradually expanding the complexity of the model of study in this research, more comprehensive data can be compiled, and thus more knowledge acquired on the many different asthmatic physiological and physical effects of *P. kudriavzevii* on an organism and its many working units. Through the subsequent analysis of these results, proteins and other molecules found in the yeast secretions can be further inspected as potential asthmatic agents and therefore linked to many other microorganisms within the biosphere. The identification of additional asthmatic agents in the biosphere has immense potential to aid in the development of more targeted asthma treatments that can combat the cellular effects of such molecules.

METHODS
Culture conditions and infection treatment

The immortalized alveolar epithelial cell line was cultured (RPMI 1640 cell culture media; supplemented with 10% FBS, penicillin, L-glutamine) until it reached the optimal confluency of approximately 85% (incubated at 37°C with 5% CO2). The *P. kudriavzevii* yeast culture (ATCC® 6258™) was cultured in YPD liquid broth media and YPD growth media agar

plates (Sigma Aldrich Y1375, Y1500 respectively). After initial culturing of the necessary model cells to sufficient confluence (~85%), the experimentation was promptly started and carried out as follows: using sterile technique, the confluent alveolar epithelial cells were lifted from one of the T75 flask's surface into suspension with the use of cell scrapers, and 2ml of the created suspension was then transferred into each well of the 6-well plates being used. For every infection treatment, 10 cell culture-treated Thermanox™ coverslips were inserted per 6-well plate (2 per well; immediately before cell seeding) for optimal attachment of cells and subsequent usage in the TUNEL and PAS staining assays. The 6-well plates were incubated under A549 cell optimal growth conditions for 24-48 hours (as needed) until proper surface attachment and ~85% confluence was achieved by the cells. *Pichia* yeast were introduced to the A549 cell line through yeast culture incubation in 6-well plate inserts (pore size 0.22 μm) for 0, 4, 6, 12, 24, and 48 hours. Non-infected controls were also incubated and assayed at these time intervals for reference. After the infection treatment, both the experimental and control cell samples were assayed using the following protocols.

Trypan blue cell viability assay

Cell viability was tested via the Trypan Blue proliferation assay (15). The materials necessary to conduct the trypan blue assay for proliferation were provided by the American Heritage School laboratory facilities. To start the trypan blue staining procedure, 100 μl cell suspension was diluted in 100 μm 0.4% Trypan Blue solution, creating a 1:1 dilution which was promptly inserted into the hemocytometer for counting. Cells were counted under an inverted microscope in four 1x1 mm squares of each chamber and thus average cells per square were determined. Appropriate formula was used to determine percentage cell viability and results were analyzed in a five-number summary and through a t-test to determine significance.

ELISA to assess cytokine levels

To start the IL-6 EISA immunoassay (Enzo Life Sciences™ catalog # ADI-902-033, Cayman Chemicals™ catalog # 501030), 100 μl of base media were added into to appropriate wells in duplicates. Additionally, 100 μl of standard, sample, or control were dispensed into their appropriate wells, with the samples being taken from the liquid supernatant of the monolayer A549 cell culture. The plate was then incubated for 1 hour at room temperature in an orbital shaker at approximately 300 rpm. After the incubation, each well was aspirated and washed using the previously prepared wash buffer, repeating the process three times for a total of four washes. The provided yellow antibody (100 μl) was added to each well and the plate was then covered with a new adhesive strip and incubated for 1 hour at room temperature in an orbital shaker. The aspiration/wash process was repeated, and 100 μl of the provided blue conjugate was added to each well and the plate was then incubated for 30 minutes at room temperature in an

orbital shaker at 300 rpm. The aspiration/wash process was repeated, and substrate Solution (100 μl) was added to each well (except the blank) and the plate was then incubated for 15 minutes at room temperature in an orbital shaker at 300 rpm. Immediately after incubation, 100 μl of Stop Solution 2 was added to all corresponding wells. Wells were thereafter read at 450 nm wavelength in a well-plate spectrophotometer and data was collected. The absorbance results yielded from the samples tested in the IL-6 (human) ELISA immunoassay (**Figure 2**) were plotted on a constructed IL-6 standard curve and used to derive a theoretical concentration of IL-6 in the corresponding samples (such concentration being dependent on where the collected absorbance measurement landed on the standard curve). The concentrations of IL-6 in the tested samples were analyzed using a t-test to derive statistical significance between groups.

PAS mucus secretion assay

Mucus secretion was tested via PAS (Periodic Acid Schiff) staining (16). Following manufacturer's procedures for the PAS staining assay (PolySciences, Inc™ 24200-1), stored coverslips were re-hydrated in distilled water and immersed in Periodic Acid Solution for 5 minutes, after which distilled water was used to rinse them (4 times). Coverslips were then covered in Schiff's Solution for 15 minutes, and distilled water was used to wash the coverslips. Coverslips were stained in Hematoxylin for 2-3 minutes, then rinsed in running tap water for 2-3 minutes. The pre-prepared Bluing Reagent was applied for 30 seconds and distilled water was subsequently used to rinse the coverslips. Finally, the coverslips were dehydrated using graded alcohols (100%,95%,80%,75%), and cells were viewed under an inverted light microscope. Subsequent data analysis was done using the cell counting feature on ImageJ software (NIH, 2018). The results from the PAS stain assay (Figure 3) were also analyzed in a five-number summary and by running a t-test with the raw results as to establish statistical significance of the acquired data. The conducted analysis yielded p-values of less than 0.05 when any of the experimental groups were compared to one another and to the 0 hr infection control group.

TUNEL assay to assess apoptosis

The TACS® 2 TdT DAB (TUNEL) diaminobenzidine kit (Trevigen™ 4810 30 K) was used in this experiment to identify and quantify the number of apoptotic cells within a cell suspension sample. Prior to the commencement of this assay, cells were fixed in 3.7% buffered formaldehyde.

To label the cells, samples were placed in 1X PBS for 10 minutes at room temperature. These were then covered with 50 μl of Proteinase K Solution and incubated for 15 to 30 minutes at room temperature, and subsequently washed 2 times in deionized water for 2 minutes each. Quenching Solution was used to completely cover the coverslips for 5 minutes at room temperature, and the samples were washed by 1X PBS for 1 minute at room temperature prior to being

submerged in 1X TdT Labeling Buffer for 5 minutes. A solution of Labeling Reaction Mix was created, and 50 µl of the solution was carefully dispensed on top of each coverslip. These were then incubated at 37 °C for 1 hour in a humidity chamber and immersed in 1X TdT Stop Buffer for 5 minutes at room temperature shortly after. 50 µl of Strep-HRP solution was carefully dispensed to cover each coverslip, and these were subsequently incubated for 10 minutes at 37 °C in a humidity chamber. After incubation, samples were washed twice with 1X PBS and drenched in DAB solution for 2 to 7 minutes before a final wash with deionized water.

To counterstain the samples and optimize visibility of the results, samples were immersed in deionized water for 2 minutes, and then for 5 seconds to 5 minutes in Methyl Green. The coverslips were then sequentially dipped into washes of deionized water, 70% ethanol, 95% ethanol, and 100% ethanol. Coverslips were turned upside down and left lying flat in the dark overnight to allow them to dry. Results were acquired and analyzed using light microscopy and digital cell-counting methods, respectively (dark brown-stained apoptotic cells/ green-stained non-apoptotic cells = percentage of apoptotic cells). The results of the TUNEL assay (**Figure 4**) were compiled into a multi-bar graph and analyzed through a five-number summary and a t-test to yield *p*-values and thus determine the statistical significance of the data obtained.

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