Sampling Virus Aerosols Using the Gelatin Membrane Filter

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Although there are already field-proven standard methods for sampling bacterial aerosols with the Sartorius Gelatin Membrane Filter, this filter has only been cautiously assessed and hesitantly used until now for collecting airborne viruses. This paper demonstrates the suitability of the gelatin filter for sampling virus aerosols by presenting the results of systematic studies using experimentally generated, static phage and virus aerosols. It also introduces a standard method for collecting viruses using a gelatin filter and for processing the filter to recover the viruses.

The impinger and impaction collector techniques already belong to the classical methods for sampling microbial aerosols. While the impinger method involves separation of the microbes based on inertial retention and filtration in a collecting liquid, the impaction collector retains microorganisms on solid agar or gelatin surfaces, again on the basis of inertia. Both methods have proved their reliability in sampling bacterial, fungus and virus aerosols in a number of tests. Moreover, impinger model AGI-30 was proclaimed the standard sampler of choice at the International Symposium on Aerobiology in 1963 in Berkeley, California [1].

Following the widespread introduction of the membrane filter – albeit with some disadvantages – for airborne microbe sampling, the membrane filter became the focus of renewed interest upon the arrival of the principle of a water-soluble gelatin filter. These gelatin filters became widely used for determining the CFU[1)] count of airborne microbes once Goettingen-based Sartorius AG started to manufacture such filters on a commercial scale. The systematic work done by Petras [2], Rotter and Koller [3] and Koller and Rotter [4] established the fundamentals for collecting airborne bacteria using the water-soluble gelatin filter.

By contrast, membrane-type filters have been used only sporadically so far for collecting airborne viruses [5–8], and the water-soluble gelatin filter was employed for the first time by Haferkorn et al. [9]. Whereas Haferkorn et al. recommend the gelatin filter "if one wishes to achieve low bacteriophage passage rates and good sensitivity in airborne bacteriophage detection using a sampler [translation of the original German quote]," Spendlove and Fannin [10] claim that the value of filters is limited for sampling virus aerosols. However, they do not present any proof supporting their assessment: "The risk that delicately structured viruses will dry out renders the filter unsuitable for long term or high-volume sampling [translation of the German quote]." Even Bogdasariyan et al. [11] express reservations: "It can be assumed that all test methods based on filtration of air through cotton, membrane and water-soluble filters are indeed suitable for the recovery of viruses that are resistant in the surrounding environment, whereas they have only limited applicability for detection of respiratory virus aerosols."

Fig. 1 T1 phage particles adhering to drop-shaped, crystallized solid material – the model of a virus aerosol particle (initial drops). Impaction of particles from an aerosol stream of an aerosolizing unit on film- and carbon-coated plates. Liquid for aerosol generation: nutrient broth diluted 1:1,000 (solids content 2 mg/ml). Scanning electron micrograph (model SEM 3-2 from the Werk für Fernsehelektronik, a Berlin-based factory for TV electronics), magnified 166,000 ×; micrograph made by Jaschhof and Fischer.

However, users are bound to completely agree in assessing the practical advantages of collecting viruses using water-soluble filters – less labor and materials needed both for repeated preparation of the equipment to perform air sampling and for processing of the filter and subsequent detection of the viruses. In addition, water-soluble gelatin filters offer the same benefits as does liquid separation: The method is virtually independent of the phage or virion concentration; a distinction can be made between individual phages or virions and their aggregates; and the phages or virions of an air sample can be simultaneously cultivated on different media.

This paper is among a series of articles that report the results and experience obtained by using Sartorius Gelatin Membrane Filters to collect viruses – T1, T3 and f2 E. coli phages and influenza viruses – recovered from defined, experimentally generated, static aerosols. In addition, a later article will discuss the first practical application of the gelatin membrane filter method for detecting airborne influenza viruses in the waiting room of a children's polyclinic.

Results with Phage Aerosols for Establishing a Standard Method

A field-proven and nearly standardized method has been introduced for sampling biological aerosols using impaction collectors and impingers. For bacterial aerosols, typical yields provided by water-soluble gelatin membrane filters have been confirmed – to a considerable extent by the initially mentioned studies performed by Petras, Koller and Rotter.

By contrast, there is a complete lack of systematic studies to determine the filter yields for virus aerosols. The objective of the tests discussed in the following was to obtain reliable data for the filtration of virus aerosols to serve as the basis for establishing a standard method.

The collector used was a Sartorius AG Air Sampler, SM 16711, with a maximum sampling rate of 2,000 l/h along with a 50-mm diameter Sartorius Gelatin Membrane Filter, type 12602-050. T1 and T3 phages served as virus models.

Choice of Air Inlet Velocity at the Filter

The inlet velocity is calculated from the quotient of the volume of air flow (cm^3/s) and the area of the filter (cm^2). An inlet velocity of 0.1–0.4 m/s corresponds to air flow rates of 7.5, 15, 22.5 and 30 l/min, respectively.

The inlet velocity is a particularly critical variable with respect to the mechanical stress on phages or microbes upon impacting the surface of collection and with respect to a drying effect to which the retained phages or microbes are exposed in the air stream. For bacteria, inlet velocities ranging from 0.1 to 0.6 m/s do not have any effect on the collection efficiency according to Petras [2]. Rotter and Koller [3] claim that "the optimal inlet velocities are probably around 0.2 m/s." Hecker et al. [12] chose 0.17 m/s, and according to Maier and Voggel [13], 0.1 m/s can be calculated, whereas for Noller and Spendlove [14], who used gelatin foam filters, an average inlet velocity of 0.06 m/s can be computed.

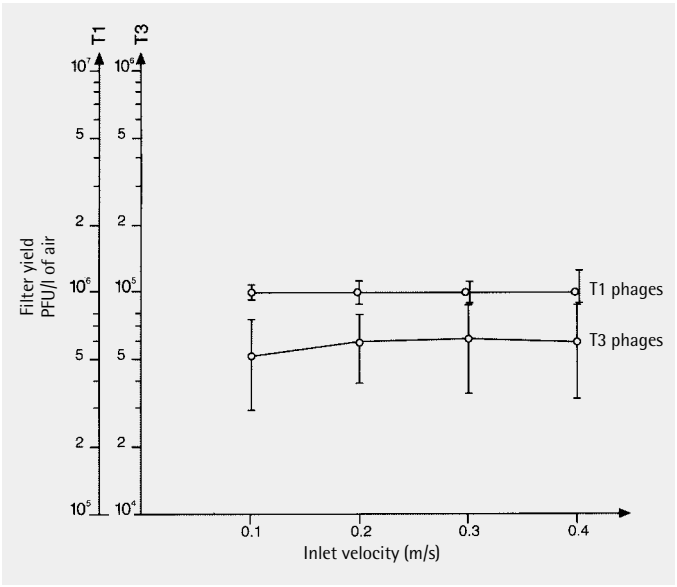

Fig. 2 Filter yields of PFU/l of air as a function of the inlet velocity for a T1 aerosol at a relative humidity between 50–55% and for a T3 aerosol at a relative humidity between 80–85%. Liquid for aerosol generation (nutrient broth) with $2.5 \cdot 10^9$ PFU/ml for T3 phages and $1.7 \cdot 10^{10}$ for T1 phages.

Figure 2 shows the results for T1 phages at 50–55% relative humidity and for T3 phages at 80–85% relative humidity. The graph shows the yields of infectious virus particles (PFU = plaque-forming units) collected on the filters per 1 liter of sampled air. In both humidity ranges, there were no differences in the recovery rates as a function of the inlet velocity ranging from 0.1 to 0.4 m/s. For the subsequent tests, 0.3 m/s was used as the standard sampling velocity.

Filter Passage in Percent as a Function of the Inlet Velocity

To determine the percentage of phages that pass through a filter, two filters, F1 and F2, were placed one in back of the other in each of the filter holders, and the number of infectious units was determined for each filter, $K_{F1} + K_{F2}$ Neglecting the number of PFUs that passed through both filters, the passage in percent (P%) was calculated based on Petras' [2] formula

$P\% = (K_{F2} \cdot 100)/(K_{F1} + K_{F2})$

For a Bacillus subtilis var. niger aerosol, Petras [2] determined a passage of 0.16% to 0.014% that decreased as the inlet velocity increased from 0.02 to 0.12 m/s. Haferkorn et al. [9] indicated values of 0.004–0.011% for a T3 aerosol; however, they did not make any reference to the air sampling velocity.

For a T3 aerosol at 80–85% relative humidity, average passages of 0.06% were obtained, independently of an inlet velocity ranging from 0.1 to 0.4 m/s (Fig. 3). Therefore, these passage percentages were five to ten times higher than those determined by Haferkorn et al. [9] for the same phages. For a T1 aerosol at 50–55% relative humidity, passages of 0.06–0.135%, and of 0.06% on average, were calculated at a standard inlet velocity of 0.3 m/s. These values matched the results obtained for T3 phages.

The data obtained for this important characteristic value are to be supplemented now with a percentage yielded by later studies using the tested maximum challenge both for the virus and the filter. The passage for particles of a T1 aerosol was approximately 0.24 ± 0.1% for a 15-minute sampling period at an inlet velocity of 1.6 m/s – which is equivalent to an air flow rate of 120 l/min – an air temperature of 30°C, and a relative humidity of 80–85%.

Table 1: Filter yields (PFU/filter) for a T3 aerosol at 80–85% relative humidity after dissolving the filters in various media. Titer of the suspension for aerosol generation (nutrient broth) $1.05 \cdot 10^{10}$ PFU/ml

Medium	Aqua ad inject.	0.85% NaCl	Phosp. buffer	1% peptone
PFU · 10^6/filter	5.64 ± 1.5	5.62 ± 0.76	6.15 ± 0.64	5.38 ± 0.68

Table 2: Filter yields (PFU/filter) as a function of the shaking time needed to dissolve the filters for sampling a T3 aerosol at 80–85% relative humidity. Titer of the suspension for aerosol generation (nutrient broth) $1.25 \cdot 10^{10}$ PFU/ml

Shaking time	0	5	15	30	60
PFU · 10^6/filter	6.52 ± 1.88	6.52 ± 1.94	6.16 ± 2.15	6.52 ± 1.86	6.69 ± 2.27

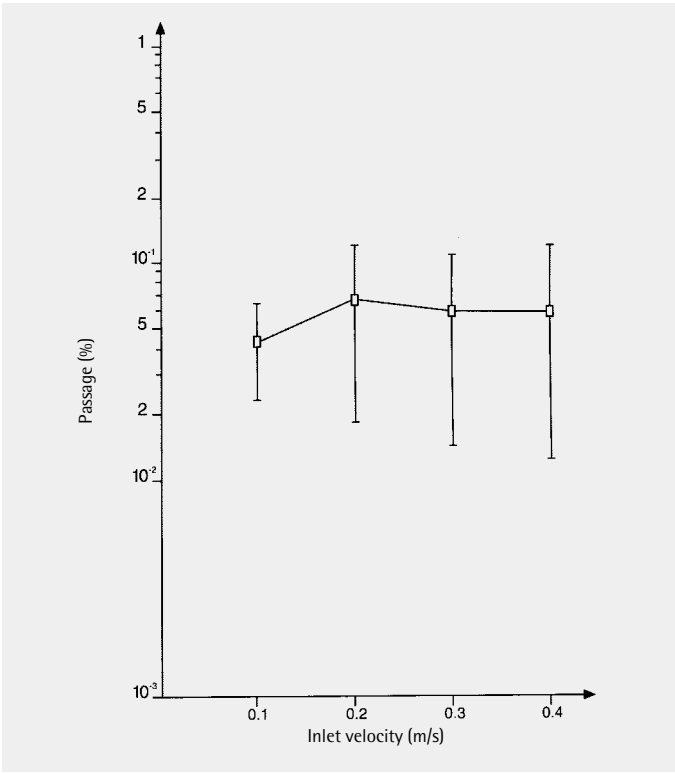

Fig. 3 Passage in percent through water-soluble gelatin filters as a function of the inlet velocity for a T3 aerosol at 80–85% relative humidity. Titer of the liquid for aerosol generation (nutrient broth) 2.5×10^9 PFU/ml.

Medium Used to Dissolve the Filter
Of the three basic techniques established for processing gelatin filters to determine the CFU count in bacterial aerosols, only one method can be used in the case of virus aerosols, at least for quantitative assays. In this method, the filter is dissolved in a suitable medium, the suspension is then shaken by mechanical means to break up virus particle aggregates, and the number of plaque-forming units is determined in or on substrates.

In the case of bacterial aerosols, Mitchell et al. [15] merely used sterile water as the medium to dissolve the filter; Noller and Spendlove [14] chose a complex solution composed of 0.5% tryptose, 1% NaCl in distilled water and an additive of 0.06% antifoaming agent. Petras [2] used 0.04% Na_2HPO_{4-} solution.

Koller and Rotter [4] found that by using physiological saline, the yield of infectious particles was 1.57 times greater than that obtained by incubating the filters on culture plates. When 1% peptone water was used, the yield over the culture plate method was 1.9 times higher.

The importance of the liquid for dissolving the filter can be expected to be secondary on account of the advantages of the gelatin nature of the filter, provided that the pH and salt concentration are held in a physiological range. The following media were used for comparison in dissolving the filters used to collect phage aerosols: Aqua ad inject. (sterile water for injection), physiological saline, m/15 phosphate buffer with a pH of 7.2, and a 1% Difco peptone solution. Each of the filters was dissolved at 37°C for 5 minutes in 20 ml of the individual solutions in a 200-ml wide-necked Erlenmeyer flask with approx. 40 glass beads of a 2.5-mm diameter. Then each suspension was shaken for an additional 5 minutes. Afterwards, there were no demonstrable statistical differences in the number of infectious units per filter as a function of the type of medium used to dissolve the filter (Table 1).

With respect to the comparative air sampling procedures – phosphate buffer served as the collecting liquid for the impinger, and a buffer base as the gelatin collection liquid for the impaction collector – m/15 phosphate buffer with a pH of 7.2 was also employed as the standard medium for dissolving the filters used in the gelatin membrane air sampling technique.

Shaking Time for Dissolving the Filters
In sampling airborne bacteria, Koller and Rotter [4] determined a progressive increase in the yield of the exposed filters after a shaking time of 60 minutes at 37°C – before this period, the filters had been left to stand in a liquid for 30 minutes to dissolve. They found that this increase in yield was up to 4.3 times higher than that obtained by incubating the filters on culture plates. Even after a shaking time of 60 minutes, the aggregates were apparently not entirely split up; however, the shaking time could not be extended on account of the possibility that the microbes might propagate in the liquid used to dissolve the filters, which was 1% peptone water.

The microbe-laden filters in the present study were each dissolved in 20 ml of phosphate buffer for 5 minutes at 37°C in a water bath without stirring or shaking. Afterwards, the solution was briefly swirled by hand (t = 0) and then shaken for 60 minutes at room temperature on a laboratory shaker. Just after 5 minutes of dissolving, the same infectious titer was present as during the 60 minutes of shaking (Table 2). Therefore, to process the exposed filters according to a standard procedure, the parameters were established as follows: a dissolving time of 5 min at 37°C in a water bath and a shaking time of 5 minutes at room temperature.

The results demonstrate an important characteristic and benefit of the gelatin filter in sampling virus aerosols. The gelatin simultaneously acts as a protective protein, a capsid, in preventing inactivation of virus particles at the glass/water and the water/air interfaces, which are formed during shaking or while air flows through liquids, as in the impinger method. Surface inactivation [16] has also been shown to be the cause of inactivation of phages in the aerosol at high relative humidities [17, 18].

Volume of Liquid for Dissolving the Filters
Reducing the liquid volume to a minimum is advantageous in terms of an additional concentration of phages, if low airborne virion or phage counts have to be detected. However, in the present study, we first had to check whether the expected increase in viscosity of the liquid for dissolving the filters would guarantee a sufficient distribution of the particle aggregates and, beyond this, actually result in the formation of suspensions of individual particles. For this purpose, shaken suspensions containing 20, 10.5 and 2.5 ml of phosphate buffer and 1:10 dilutions of these suspensions in nutrient broth were vigorously mixed and then titrated using a 5-ml pipette.

As expected, a definite increase in the viscosity was determined in the 5-ml and the 2.5-ml samples, respectively. Surprisingly, however, this increase did not have a demonstrable effect on the distribution of the plaque-forming units (Fig 4). When the number of infectious units in 20 ml of the shaken suspension was equated with 100%, 96.05% PFU was obtained on average for 10 ml, 100.2% for 5 ml and 94.5% for 2.5 ml. The titration of the 1:10 dilutions, which translated to a 1:10 decrease in viscosity, did not result in any additional demonstrable increase in the PFU yield over that of the original suspension (Fig. 4).

As the standard volume, 20 ml of liquid were first used. In later studies, 5 ml of phosphate buffer with a reduced number of glass beads proved to be increasingly practical for dissolving the membrane filters.

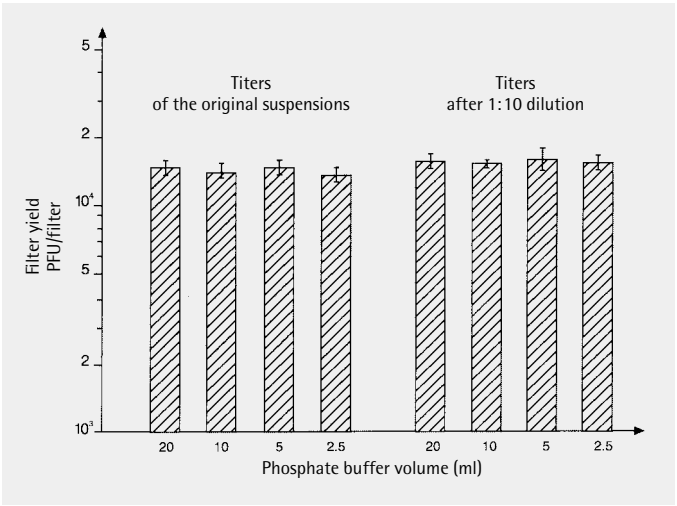

Fig. 4 Filter yields (PFU/filter) as a function of the volume of original phosphate buffer suspensions and of 1:10 dilutions of these suspensions, both types of which were prepared from dissolved filters used to sample T1 aerosol at 50–55% relative humidity. Titers of the liquid for aerosol generation (nutrient broth) $1.3 \cdot 10^7$ PFU/ml

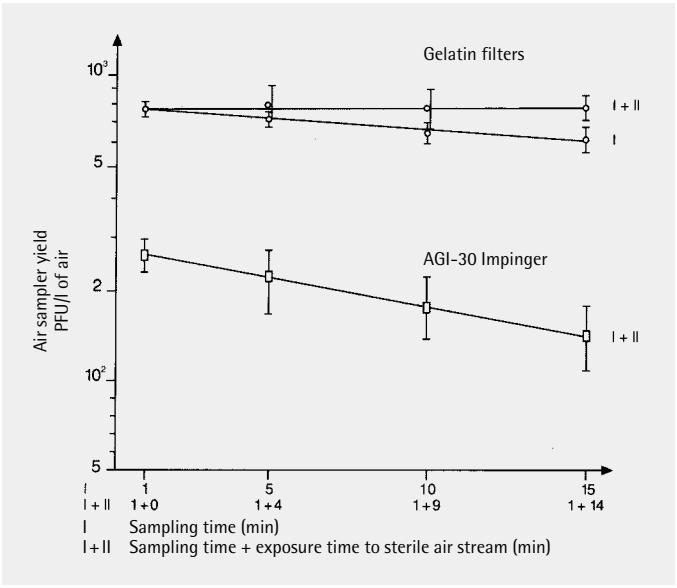

Fig. 5 Effect of the sampling time on the collection efficiency. Titers of the aerosol suspension (nutrient broth) $1.7 \cdot 10^7$ PFU/ml. I: Number of PFUs/l of air during parallel sampling procedures carried out at four locations for different lengths of time. I + II: Number of PFUs/l of air during parallel sampling procedures carried out at four different locations but for the same time of 1 minute each and for different times of exposures of the filters to sterile air streams with 50–55% relative humidity at an inlet velocity of 0.3 m/s following the aerosol sampling procedures. 1-min sampling using an impinger at a rate of 12.5 ml/min and subsequent exposure to a sterile air stream at a 50–55% relative humidity and a rate of 12.5 l/min for 4.9 and 14 minutes, resp.

Effect of the Sampling Time

The sampling time is also significant in detecting low virion or phage concentrations in aerosols. Prolonging the sampling time is problematic in view of the increasing stress which the air stream places on biological particles already collected on the filter. Data for collecting airborne bacteria are provided by Rotter and Koller [3]. Equating the results for a 1-minute sampling period with 100%, the yields of microbes recovered after a 5-minute sampling period were only 88% and after 60 min 55%. Petras [2] determined a strong drop among the yields of colony-forming units after sampling airborne microbes in a rabbit hutch for 1 min, 2 min and 4 min, respectively. Sampling periods of 8, 16 and 32 min yielded increasingly constant values. Koller and Rotter [4] studied the effect which different periods of subsequent sterile air sampling procedures had on the stability of microbes already collected on gelatin filters. Sterile air was drawn through gelatin filters for 5 min. After this initial period, there was a marked decrease in the number of microbes to approx. 85% of the initial values. Following a 60-minute period of sterile air sampling, the drop in number to 75% was insignificant. Therefore, 5 min can be considered the maximum sampling time.

For a T1 aerosol at 50–55% relative humidity, the number of infectious particles per liter of sampled air decreased following uninterrupted sampling at an inlet velocity of 0.3 m/s. This decrease was 82% compared with the reference value for a 1-min sampling period (Fig. 5, I). The results of exposing the filters to a sterile air stream following actual aerosol sampling confirm the assumption that this difference in infectious particles recovered cannot be attributed to inactivation caused by prolonged exposure to the air stream but to a considerable degree to the aging of the aerosol during sampling – dilution of the aerosol as the cause of such inactivation was able to be neglected by way of calculation. Drawing sterile air for 4.9 and 14 minutes, resp., at a 50–55% relative humidity and an inlet velocity of 0.3 m/s, through filters previously exposed to virus aerosol streams for 1 min did not result in any decrease in the number of infectious particles compared with the control filters not exposed to a sterile air stream after aerosol sampling (Figure 5, I and II).

With the impinger by comparison, a definite inactivation of the retained T1 particles was discovered under the conditions of subsequent sterile air sampling, which corresponded to a prolonged aerosol sampling time. A sampling time of 1–2 min under standard conditions was specified.

Standard Procedure for Sampling Virus Aerosols

As the result of the test studies presented, the following procedure is recommended as the standard method for detecting viruses in aerosols using the Sartorius Gelatin Membrane Filter:

I. Collecting the Viruses

Air inlet velocity at the filter: 0.3 m/s, equivalent to 22.5 l/min
Sampling time: 1–2 min.

II. Processing the Filters

for dissolving and shaking the filters in the appropriate liquid. Liquid for dissolving the filters: m/15 phosphate buffer, pH 7.2
Flask for shaking the suspension: 200 ml wide-necked Erlenmeyer flask with 10–40 glass beads with a 2.5 mm diameter.
Volume of the liquid for dissolving the filters: 20 → 5 ml.

Dissolving temperature: 37°C in a water bath.
Dissolving time: 5 min.
Shaking time for the dissolved filter:
5 min at room temperature.
These parameters are for a 50-mm diameter filter and can be applied accordingly to 80-mm filters. However, these larger diameter filters must first be broken apart in order to be placed in the flask specified.

Koller and Rotter [4] indicate 150 colony-forming units (CFU)/m^3 as the lower detection limit for airborne bacteria collected on gelatin filters. They base their calculation on a five-minute sampling period at 0.4 m/s. This would still yield 30 CFUs/filter when the filters are directly incubated on culture plates.

In the case of virus aerosols, direct cultivation by allowing the gelatin filter to melt on the microplates is not recommended as a standard method. For phage aerosols, the plaques formed are cloudy and not sharply defined; therefore, quantitative assay is not guaranteed. In the case of pathogenic viruses, the possible influence of the gelatin concentration on the cell system and its effects on virus replication are additional drawbacks. The quantitative lower detection limit for viruses in aerosols under standard sampling conditions must therefore be raised by several powers of ten to be in keeping with the method. For a virus titration in an incubated egg, for example to determine EID_{50} endpoints according to Reed's and Muench's method [19], approx. $4.0 \cdot 10^4$ infectious particles/m^3 of air are required as the minimum concentration. In 0.2 ml of the suspension containing the dissolved exposed filter, the average volume needed to infect an incubated, embryonated egg, this yields ten infectious units. This quantity guarantees a positive reaction in the entire group of reagents. For phage aerosols, this detection limit is shifted to an even higher level of approx. 10^5 PFU/m^3 of air. If 0.8 ml of the gelatin/virus suspension is used per agar overlay plate, 135 PFUs per plate are obtained.

For 80-mm diameter gelatin filters, lower detection limits of approx. $1.4 \cdot 10^4$ or $5.0 \cdot 10^4$ infectious units/m^3 of air are calculated for the same inlet velocities as for 50-mm filters during sampling, on account of the larger effective filtration area.

Outlook

The author's articles due to be published at a later date will report on studies comparing the collection efficiencies of the impinger, impaction collector and the gelatin filter for sampling virus aerosols. Moreover, the articles will discuss how the potential of gelatin filter sampling can be used to decrease the lower detection limit by several powers of ten for viruses as well.

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