Correlation between foot-and-mouth disease virus antigenic mass, titer and immune response in vaccinated sheep

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A B S T R A C T

The antigen mass load of foot-and-mouth disease virus (FMDV) is one of the critical clues to produce a potent vaccine. In the current work, the quantification of the virus 146S antigen content was described and the relationship between antigen mass in the vaccine and the immune response to FMDV in vaccinated sheep were investigated. It was found that FMD vaccine containing at least 1.5 µg/2ml (small ruminant dose) or 3 µg/2ml (cattle and buffaloes dose) from each serotype of FMDV 146S particles and virus titers 6.55-7.3 log10 TCID50/ml gave protective immune response against FMDV. Moreover, relying on the antigenic mass coupled to ultrafiltration of the virus was showed to be of better quality and potency than depending on the volume of the virus suspensions in the formulation of FMD vaccine; however, the former technology needs with standing more cost and effort .

Keywords: Foot-and-Mouth disease virus; 146S, antigen mass; ultrafiltration

(http://www.bvmj.bu.edu.eg) ***conference issue*** **(BVMJ-28(2): 12-19, 2015)**

1. INTRODUCTION

Foot and mouth disease virus (FMDV) is a highly contagious viral disease of biungulates animals causing loss of appetite, lameness, drop in milk production and loss of body weight with high morbidity in adult and mortality in calves (OIE, 2012). Vaccination against the virus is still the applied policy in most developing countries e.g. Egypt in order to control the disease. Protection of livestock vaccinated by FMD vaccine from incursion of the virus depends mainly on two keys: the vaccine antigen content, besides, the percentage of homogeneity and relatedness of vaccine virus to the wild virus. The antigenic content of FMDV evokes neutralizing antibodies and is depend on the presence of intact capsid, as either the whole virus particle with a sedimentation rate of 146S or the natural empty capsid which sediments at 75S (Rweyemamu et al., 1989).

Historically, formulation of FMD vaccine depended on the volume of the virus suspensions that were harvested from cell culture inoculated by virus. Estimation of the virus content was done by virus infectivity on cell culture e.g. BHK-21 in order to know the titer of the virus or by complement fixation test to define the complement fixing unit (Terpstra et al., 1976). The former antigen titer assays had some limitations. Recently, large differences in sensitivity of cell cultures used for virus isolation studies between five participating European reference laboratories were reported (Ferris et al., 2006). Besides, the titer of the same FMDV sample, stored at -80 ºC in a single use aliquot, diverged from BHK cells batch to another. This might be due to the difference in the handling technique of the operators, in addition to, the reduction in the susceptibility of cells to the virus due to sub-culturing and aging coupled to using of various batches of sub-culturing supplies e.g. trypsin, growth media, newly born calf serum, etc. Moreover, BHK cells, the main cells that our lab depend on in all purposes

including production of the vaccine viruses and its evaluation, showed less sensitivity to FMDV detection by about 100-1000 fold than the PCR assays (Azab et al, 2012). Complement fixation test (CFT) has several disadvantages, among them: (i) The CFT is relatively insensitive; (ii) Pro- and anti-complementary activity can invalidate the CFT; (iii) In the micro-CFT, results are objectively read and can vary between test operators; (iv) the CFT is relatively extravagant on the use of type-specific antisera (Ferris and Dawson, 1988).

Since the last four decades, quantification of FMDV 146S particles has been the main international standard assay for formulating vaccines containing predetermined levels of the antigen. It is known that the immunizing ability of FMDV vaccines is associated primarily with the quantity of the intact 146S particles in vaccine (Terry et al, 1982; Shirai et al., 1990 and Spitteler et al., 2011). Fractionation and quantification of FMDV particles are usually done by density gradients to separate particles depending on two alternative operations: (i) size differences (isokinetic sedimentation centrifugation) through a linear gradient, usually of sucrose; and (ii) densities difference (isopycnography sedimentation, which stands for equilibrium density gradient centrifugation) through a salt gradient e.g. caesium chloride, CsCl (Barzilai et al., 1972).

The Measurement assay of 146S antigen content of FMDV has not well been established in the developing countries, especially, in the African Middle East countries' labs where limited investigations were performed on the assay. Moreover, the faced troubleshooting and obstacles by the investigators in their labs' were usually not pointed out (Hegazi, 2000; Ali et al., 2010 and Hiam et al., 2013). In this paper, quantification of FMDV 146S particles by sucrose density gradient centrifugation was described. In addition, preparation of FMD vaccine batches with different 146S antigen dose were prepared and injected in sheep to study the relationship between antigen mass in the vaccine and the immune response to FMD

2. MATERIALS AND METHODS

2.1. Virus and Samples

FMDV O/EGY/2009 iso1, A/EGY/2009 iso-Cai and SAT2/EGY/H1Ghb/2012 local strains of serotypes O, A and SAT2, respectively (EL-Shehawy et al., 2011 and 2014) were passaged on monolayer BHK-21 cells for seven times to be ready to prepare the following virus samples. The viruses were collected after freezing and thawing to prepare FMDV samples. The supernatant of the viruses harvest were purified by 8 % polyethylene glycol (PEG) 6000 (Merck, Germany). In brief, the viruses were stirred with PEG for 120 min at 4 ºC, followed by sedimentation of PEG adsorbed virus particles by centrifugation at 4500 rpm for 30 min at 4 ºC. The particles were eluted from the PEG sediment using Tris-bufferd saline (TBS) that was consisted of 8 g NaCl, 0.2 kCl, 3 g Tris base in Liter of DDW, pH 7.4 (Sambrook and Russell, 2001). The virus particles were liberated in the buffer and collected by two successive centrifugation of the suspension at 4500 rpm for 15 min at 4 ºC. The PEG treated viruses were sterilized by Syringe Filter (0.2 µm, 26 mm) to have FMDV PEG samples. Each virus sample was divided into two aliquots; one was used for direct preparation of trivalent FMD vaccine that was termed virus suspensions volume (VSV) batch of the vaccine, while the other was subjected to ultra-filtration. The virus infectivity titration on BHK-21 cells was performed on the samples before and after PEG treatment for the satisfaction of the purification procedures. For further purification and concentration of the PEG treated culture supernatants, ultra-filtration using Centricon Plus-70 filter device, 30K with regenerated cellulose membrane (Millipore, USA) was implemented according to the manufacturer's instructions in order to prepare FMDV PEG ultra-filter positive control samples. The controls were

treated by 10 µg/ml of ribonuclease A (Sigma-Aldrich, USA) at 25 ºC for 1h, and chilled at 4 ºC to obtain FMDV PEG ultra-filter Rnase samples. The three samples: (i) FMDV (ii) FMDV purified by PEG and (iii) FMDV subjected to PEG and ultra-filtration with ribonuclease treatment, plus the positive control samples within the former serotypes and the negative control samples were exposed to sucrose gradient centrifugation and analysis. The negative control was healthy non-infected BHK-21 cells .

2.2. Estimation of FMDV 146S antigen mass

The samples and the controls were exposed to protein quantification by sucrose density gradient and ultracentrifugation using ultracentrifuge (Kontron, Italy). Sucrose density gradients (10-40%) in PBS pH (7.2-7.6) were performed in translucent non puncturable thick wall polyallomer (polya.) 5 ml tubes. One ml of each sucrose density was applied on the denser layer from the bottom of the tube to the top. The sucrose density gradient tubes were each top layered with 200 µl of each sample except FMDV PEG ultra-filter Rnase sample with 100 µl, whereas with 50 or 100 µl of the positive control sample. The samples were ultracentrifuged for 45 min at 45,000 rpm. The spined tubes was UV scanned for the sedimentation coefficient of the whole virus particle on the programmable density gradient fractionation system. The system composed of syringe pump (Brandel, USA), density gradient flow cell (Brandel, USA), Optical Unit UA-6 Detector with 254 filter (Teledyne Isco, USA) and Foxy JR Fraction Collector (Teledyne Isco, USA) and chart recorder. For scanning samples, the top holder grasped the centrifuge tube by the soft rubber collar and the UV detector with 254 nm filter produced a continuous absorbance profile of the gradients. Displacing of the gradient samples was performed using 55% sucrose chasing buffer with the aid of the tube holder cannula from the top. Above the centrifuge tube, 5 mm flow cell was mounted to allow large flow passage and UV scanning of the gradient samples. Chasing Buffer was injected by syringe pump in rate of 1 ml/min. UV detector was adjusted at sensitivity 0.5, peak separator 1, chart speed 60 cm/h, noise filter 0.5. The UV detector drew different curves representing different sedimentation coefficient fractionated particles. The area under the 146S peak was calculated and used to determine the concentration of the antigenic mass in the samples using the following formula (Rweyemamu et al., 1989). Where: FR = flow rate in ml/min, PA = area under the peak in cm2, FSD =full-scale absorbance optical density unit setting, S =speed of the chart recorder in cm/min, PL =path length of the flow cell in cm, E =extinction point for FMDV, i.e. 72 at 254 nm, W =sample volume (ml) applied to gradient, C = concentration factor.

2.3. Vaccine formulation and animal vaccination

FMDV PEG ultra-filter positive control samples of serotypes O, A and SAT2 were exploited for preparation of trivalent FMD vaccine batches with different pre-estimated 146S antigen doses. Four batches (B1, B2, B3 and B4) of trivalent FMD serotypes O, A and SAT2 vaccine were formulated by the standard concentration of its component except the antigen content. The four batches contained different 146S antigen concentrations of each serotype: 0.33, 1, 3 and 9 µg / 2 ml vaccine dose (cattle dose) for B1, B2, B3 and B4, respectively. Each 146S antigen dosage was added to fixed volume of Montanide ISA 206 (Seppic, France) and the vaccine batches doses were completed up using sterile PBS. The vaccine batch termed VSV was prepared using 1.5 ml volume of the virus suspension /3ml vaccine dose. The vaccine batch VSV was a monovalent FMD vaccine of serotype O. The previous batch was increased to 3 ml vaccine dose to be capable to mix the whole 1.5 ml of the virus suspension with the oil adjuvant and

therefore 1.5 ml S/C dose was used to vaccinate small ruminants. The ratio of the aqueous antigen to the oil adjuvant was 50:50 for all the current vaccine batches .

Fourteen male sheep (6–9 months old) free from antibodies against FMDV O, A and SAT2 were used to evaluate the different antigenic mass of the freshly prepared vaccine batches. Animals were classified into four groups that were named G1, G2, G3 and G4, which vaccinated with batches B1, B2, B3 and B4, respectively. Each group was consisted of three animals. The remained two animals compose a group viz. VSV group that was used as positive control to be injected by VSV vaccine batch. Individual animal within each group received vaccine dose of 1 ml S/C except VSV group that vaccinated by 1.5 ml from the corresponding vaccine batch. Serum samples were periodically collected and checked for the immune response against FMDV using virus neutralization test (VNT). Determination of humeral seroconversion by VNT was carried out onto BHK-21 cells as prescribed for international trade. Titers were expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells were protected (Kärber, 1931). A titer of 1/45 or more of the final serum dilution in the serum/virus mixture was regarded as positive. A titer of less than 1/16 was considered to be negative. Titers of 1/16 to 1/32 were considered to be doubtful, and further serum samples were re-tested; results were considered positive when the second sample had a titer of 1/16 or greater (OIE, 2012). VNT geometric mean titers (GMT) of each vaccinated animal group were calculated. The GMT were estimated versus the mentioned Egyptian local strains within serotypes O, A and SAT2 .

3. RESULTS

Virus infectivity titration on BHK-21 monolayer cells was determined as log10 of inverse proportion of the last virus dilution gave 50% cytopathic effect of wells. The virus titers before and after PEG treatment (1x) of serotype O were 7.3 and 6.55 log10 TCID50/ml, respectively. Approximately the same titers were obtained with both serotypes A and SAT2. Protein quantification of virus samples, positive and negative controls was implemented by sucrose density gradient. Negative control resulted in high absorbance peak at the beginning of displacement of the gradient sample inside the flow cell (Fig. 1). The positive control illustrated that peak obtained before the last one (1 ml from the bottom of the gradient in the 5 ml centrifuge tube) was proposed to be respective to the purified concentrated virus 146S (Fig. 2). The purified concentrated positive control sample viz. FMDV PEG ultra-filter sample was measured five times at three successive days and resulted in mean average of 146S concentration equal to 2 µg/ml in the original sample (FMDV sample) or 100 µg/ml in the purified concentrated (100x) virus. Various investigated samples showed curves with different peak at the same sedimentation position as that of the positive control (Fig. 3). Roughly, the same previous antigenic mass of 146S concentration was obtained with both serotypes A and SAT2. In order to prepare different 146S antigen content of 0.33, 1, 3 and 9 µg / 2 ml vaccine dose, micro-liters of 3.3, 10, 30 and 90 / 2 ml vaccine dose from the purified concentrated samples of each serotype were loaded in FMD vaccine batches B1, B2, B3 and B4, respectively .

The GMT results of antibody detection assay varied between the vaccinated groups but did not show significant differences within each group either examined against serotypes O or A or SAT2. Thus, the expressed geometric mean titers represented the mean titers of FMDV serotypes O, A and SAT2 (Fig 4). The antibody level reached the protective level (1.65 log10) within 3-4 weeks in groups 3 and 4, which received 1.5 µg and 4.5 µg of 146 S, respectively. While groups 1 and 2 that were injected by vaccine contained

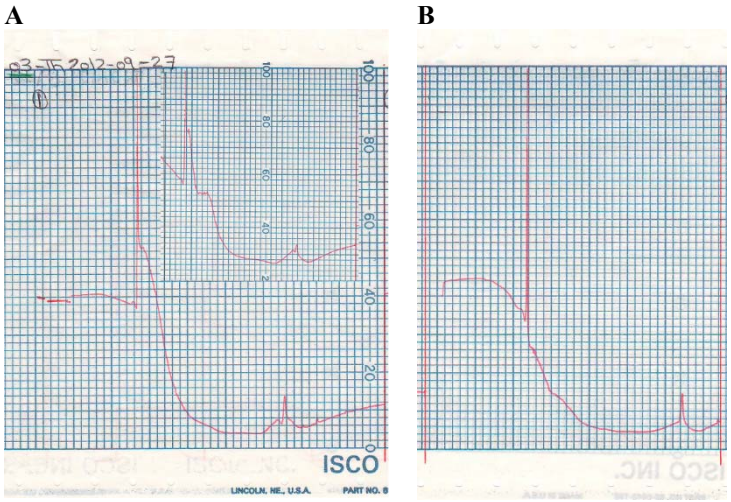


Fig. 1. UV scanning of 200 µl of healthy non-infected BHK cells negative control after sucrose gradient centrifugation. The top of the gradient was to the left of the fig. **(A)** Gradient analysis of duplicate control (main and insets charts) that was top layered in *two* centrifuge tubes on the *same* day. **(B)** Gradient analysis of control that was top layered in *one* centrifuge tube on *another* day.

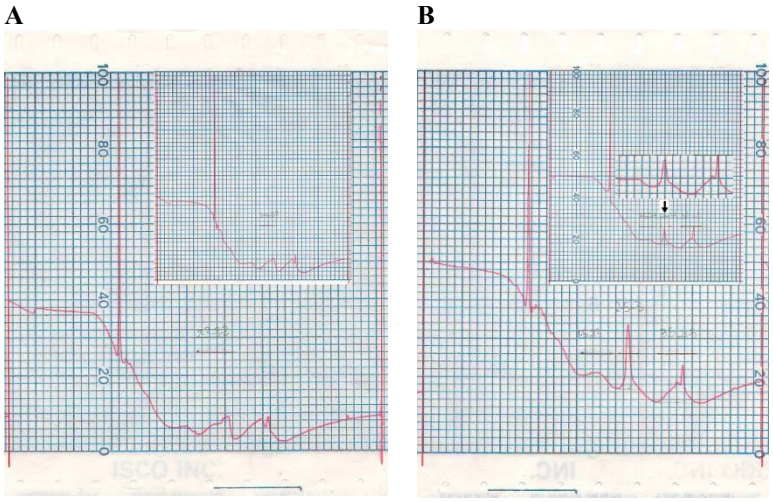
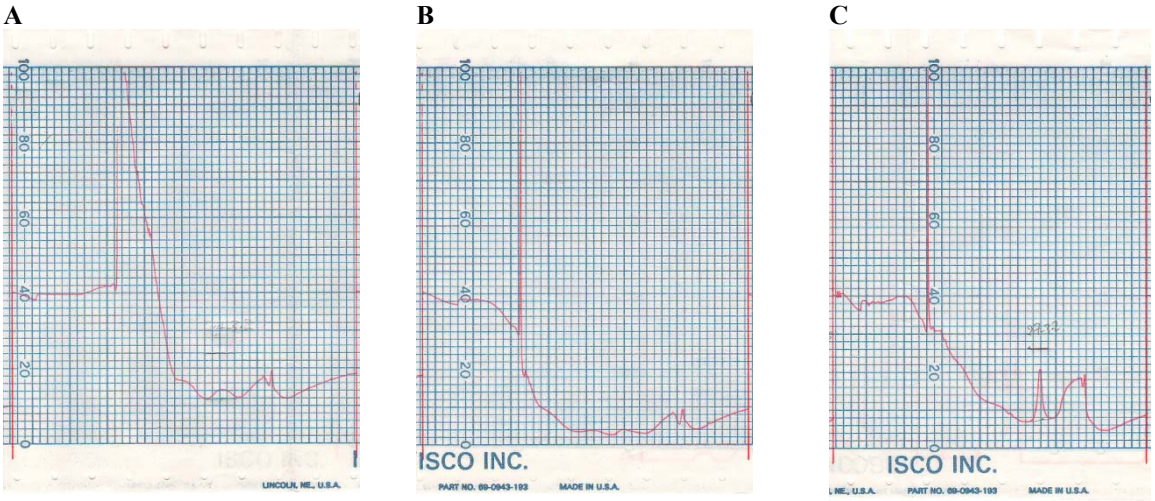


Fig. 2. Continuous absorbance profile of FMDV positive control subjected to PEG and ultrafiltration. **(A)** Gradient analysis of 100 µl of duplicate control (main and insets charts) that was top layered in *two* centrifuge tubes on the *same* day. **(B)** The insets and main charts were the gradient analysis of 50 µl and 100 µl, respectively, of the duplicate control that was top layered in *two* centrifuge tubes on *another* day.

Fig. 3. Sedimentation profile of various samples in sucrose gradients. **(A)** 200 µl of FMDV **(B)** 200 µl of FMDV purified by PEG **(C)** 100 µl of FMDV subjected to PEG and ultrafiltration with Rnase treatment.



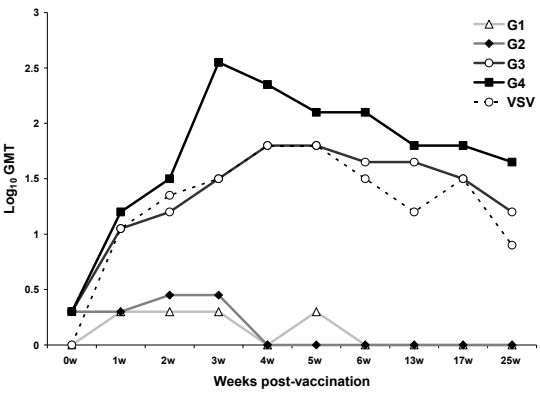


Fig 4. Antibody geometric mean titers of each animal group (G) vaccinated with different FMDV 146S antigen dose. Each animal in G1, G2, G3 and G4 received 1 ml from the trivalent vaccine containing 0.33, 1, 3 and 9 µg of the146S from each serotype /2 ml vaccine dose, respectively. The virus suspensions volume (VSV) animal group was injected by 1.5 ml from the monovalent vaccine batch VSV that contained 1.5 ml volume of the virus suspension /3ml formulated vaccine dose.

0.165 µg and 0.5 µg of FMDV 146S did not exhibit considerable sero-conversion against the virus along the period of blood sampling. The protective level for group three was at the border whereas for group four was the satisfactory antibody titers cable to protect animals without the fear of virus immunity escape. The VSV positive control animals group (injected by VSV vaccine batch) showed border positive antibodies level to FMDV, which was compatible with that of animals in group 3.

4. DISCUSSION

Sucrose density gradient fractionation depend on applying the sample to the top of the gradient and centrifuging particles will travel to that point in the gradient which is equal to their own density. At this point, they will stop and be separated from other dissimilar particles. The one large curve seen in positive and negative samples was top fractions that could be removed by sucking off 1 ml from the gradient top and discarding in order to prevent contamination of 146S peak with high extinction material (Barteling and Meloen, 1974). Sedimentation coefficients (s) of a particle are used to define its behavior in the sedimentation process in response to an external force such as centrifugation force. Sedimentation coefficients are the sedimentation velocity per unit of centrifugal force and are expressed in Svedberg units (S). Svedberg is a unit of sedimentation velocity where larger particles tend to sediment faster than small ones and therefore have higher Svedberg vales i.e. heavier particles travel to the bottom of the tube, while lighter ones will go to the top. The suggested peak of 146S in fig 2 (A) and (B) were varied in the curve configuration, which might be attributed to the difference in the cautious handling with the centrifuge tube during fixation in the rubber collar in order to introduce the gradient samples to the UV scanning. However, the results of quantification of 146S from fig 2 (A) and (B) were approximately equal. Raising the density gradient samples through the scanning cell for analysis was done by the available system in our lab using the tube holder cannula and syringe pump. The cannula was used from the top to introduce the chasing solution for the displacement of the gradients and it gave variable 146S quantification results within the same sample but with similar range with the repeatability. The variance of results might be due to the cannula interrupted the gradient layers, in addition to, it might carried on its tip protein particle from high extinction material from the top fractions. The previous would result in changing the position of sediment particle in the tube coupled by altering the peaks area of various scanned fractions. In consequence of that, tube piercing from the bottom using transparent puncturable thin wall polya. tubes and the introduction of chasing buffer by Tris peristaltic pump is recommended as mentioned by the density gradient fractionation system manufacture's

instructions, where raising the gradient intact by bulk flow minimize broadening of zones (preserves the strata) of separated particles in density gradient fractionation that lead to improve the resolution. Moreover, by employing bulk flow, it would be possible to achieve flow in which the center velocity and the edge would be the same and no differential movement (lamina flow) would be detected. The assay of density gradient centrifugation of viral samples consumed about five hours for six samples from the preparation of gradients till the end of UV scanning of the samples' fractions. It was laborious for few samples. Therefore, density gradients could be formed with automatic equipment e.g. Isco Model 160 Gradient Former that is provided with Isco's programmable Density Gradient System in order to improve precision as well as save time and effort of quantification of FMDV 146S immunizing particles. Furthermore, serological usage of coating ELISA plates with the monoclonal antibodies (Van Maanen and Terpstra, 1990) could be concurrently employed with the physical method using analytical ultracentrifugation to quantify 146S antigen of FMDV.

The immune response determination assay results for animal groups 1 and 2 injected by 0.165 µg and 0.5 µg of FMDV immunizing antigen, respectively, were disappointed. Nevertheless, sero-conversion was recorded in the other groups. Antigen mass equal to 1.5 µg for small ruminants (3 µg for large ruminants) was the cut off for the positive immune result to FMDV. However, the defined cut off could be altered in future, if more investigations will be performed to exclude any foreign protein particles that might be interfere with the current 146S measurements of FMDV. In the context of this, it is preferable to use vaccine contain 4.5 µg of FMDV 146S antigen content of each serotype for small ruminants (9 µg for large ruminants) to produce good level of FMDV antibodies. Using the ultrafiltration of the virus with 146 particles estimation instead of the volume of the virus suspension has the following privileges. It give the easiness to formulate polyvalent FMD vaccine with heterologous strains within different serotypes, decrease the volume of the vaccine dose and load sufficient antigen content without its wasting in order to produce more potent vaccine.

FMD vaccine containing at least 3 µg/2ml (cattle and buffaloes dose) or 1.5 µg/2ml (small ruminant dose) from each serotype of FMDV 146S particles gave in-vivo protective immune response against FMDV. Nevertheless, there were some obstacles faced during the quantification of the antigen mass. Therefore, more investigations are indispensable to enhance the proficiency of FMDV 146S estimation in our lab, especially with the application of the vaccination policy in the country to face the two new exotic serotypes that invade the country within 2006-2012 with the possibility of incursion of new serotypes. Furthermore, it is a promising to rely on the antigenic mass coupled to ultrafiltration of the virus instead of the volume of the virus suspensions in the formulation of FMD vaccine with enduring more cost and labor in order to improve the quality and potency of the vaccine.

5. ACKNOWLEDGMENTS

The authors are thankful to the Egyptian Veterinary Serum and Vaccine Research Institute (VSVRI) for funding the research, as well as, to Prof. Dr Laila EL-Shehawy for providing the ultra-filter.

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