

Scanning Electron Microscopy and Transmission Electron Microscopy of Mollicutes: Challenges and Opportunities

C. T. K.-H. Stadtländer*

Greenville Hospital System/Clemson University Biomedical Cooperative, and Department of Microbiology, Clemson University, Clemson, SC 29634, USA

Electron Microscopy (EM) is an important viewing technique for the study of microorganisms. More specifically, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are extremely useful tools for the ultrastructural examination of prokaryotic cells as well as for the study of the interaction between bacterial pathogens and host cells. Perhaps one of the most interesting microbes to study are the mollicutes, also known by their trivial name mycoplasmas. The name mollicutes means soft-skin organisms indicating that these microbes lack a rigid cell wall. Mollicutes are particularly difficult to handle for SEM and TEM investigation because their cell body can be easily deformed and their cytoplasmic membranes can rupture when subjected to the numerous specimen preparation steps required for SEM and TEM investigation. This paper describes the challenges and opportunities of studying soft-skin microorganisms with EM techniques.

Keywords Electron Microscopy (EM); Scanning Electron Microscopy (SEM); Transmission Electron Microscopy (TEM); Light Microscopy (LM); mollicutes; mycoplasmas; biological specimens; sample preparation; artefacts

1. Electron microscopy

Electron Microscopy (EM) can be defined as a specialized field of science that employs the electron microscope as a tool and uses a beam of electrons to form an image of a specimen [1, 2]. In contrast to light microscopy (LM) which uses visible light as a source of illumination and optical (glass) lenses to magnify specimens in the range between approximately 10 to 1,000 times their original size, EM is operated in the vacuum and focuses the electron beam and magnifies images with the help of electromagnetic lenses. The electron microscope takes advantage of the much shorter wavelength of the electron (e.g., $\lambda = 0.005$ nm at an accelerating voltage of 50 kV) when compared to the wavelengths of visible light ($\lambda = 400$ nm to 700 nm) [3]. When the accelerating voltage is increased in EM, the wavelength decreases and resolution decreases. In other words, increasing the velocity of electrons results in a shorter wavelength and increased resolving power [3].

Research development of electron microscopes began in the 1920s. Under the guidance of Max Knoll, Ernst Ruska began work on the development of electron lenses at the Technical University of Berlin, Germany, in 1928 [4, 5]. His work was fundamental for the subsequent creation of an electron microscope in that these lenses were needed to channel the electrons of the beam. The first functional transmission electron microscope was developed in the early 1930s by Ruska who constructed a two-stage electron microscope with three magnetic lenses, condenser, objective, and projector [5]. Early electron microscopic studies were primarily focused on the study of the optical behaviour of electron beams under various conditions. Thus, no biological applications were initially envisioned. However, it soon became clear that the superior magnifying power of an electron microscope could be applied to the study of the structure of various specimens, including those from plants, animals, microorganisms, and viruses [5, 6]. EM is considered today by many scientists as one of the most significant and useful developments for the ultrastructural investigation of specimens in the life sciences as well as in physics and material science [5]. Because of the broad applications of the electron microscope to the fields of

* Current address of author: University of St. Thomas, St. Paul, MN, USA; e-mail: ctstadtlande@stthomas.edu

biology, medicine, and material science, Ruska received the prestigious Nobel Prize in Physics in 1986 for his fundamental work in electron optics and for the design of the first electron microscope [1, 4].

The two basic types of electron microscopes are the scanning electron microscope and the transmission electron microscope. Although both types were invented within the same decade (the scanning electron microscope was invented by Manfred von Ardenne in 1938 [5]), they differ fundamentally in their uses. In brief, the scanning electron microscope generates an image with the help of secondary electrons that gives the viewer the impression of three dimensions, while the transmission electron microscope projects electrons through an ultrathin slice of the specimen and produces a two-dimensional image. There is also a third, less used type of electron microscope, the scanning transmission electron microscope which has features of both and uses a scanning electron beam to penetrate thin specimens. Depending on the instrument used, specimens can be magnified roughly between 10 and 100,000 times in scanning electron microscopes and between 500 to 500,000 times in transmission electron microscopes [3]. Extreme high magnifications above 200,000 are rarely used by biologists [7]. However, the relatively high magnification range of both basic types of EM allows investigators to detect in specimens much greater detail than in those examined by LM. This makes electron microscopes extremely valuable tools for the ultrastructural examination of any kind of object, but in particular for specimens of very small size.

Conventional electron microscopy is used today in many research laboratories. These microscopes have now been adapted to operate under the guidance of computers which makes sample viewing, photograph storage and labelling, as well as micrograph analysis easier for the investigator. There are also numerous specialized instrument developments in the field of EM in recent years, such as the creation of Low Energy Electron Microscopy (LEEM) that operates at low accelerating voltages (1-100 V) and is used primarily for the in-situ real-time study of dynamic processes in materials by reflection of low-energy electrons; Intermediate Voltage Electron Microscopy (IVEM) is used at an accelerating voltage of 300-400 kV for imaging of thick sections of biological and material specimens; High Resolution Electron Microscopy (HREM) operates at the limit of resolution of the instrument to obtain atomic-resolution images and chemical information from electron energy-loss spectroscopy; finally, Environmental Electron Microscopy (EEM) allows the examination of unprocessed and hydrated specimens in their native state [2, 5].

There is plenty of evidence in the published literature that EM has significantly contributed to our understanding of the ultrastructure of a variety of specimens, including those of pathogenic and non-pathogenic bacteria [6]. Without the electron microscope, we would not have the current knowledge about Gram-positive bacteria, Gram-negative bacteria, and the mollicutes. However, the preparation of prokaryotic specimens, in particular of mollicutes, poses major risks. The reason is that specimens for EM are subjected to a multitude of preparation steps in order to optimally preserve and sufficiently prepare them for viewing in vacuum with the help of electrons. Preparatory steps of specimens include, for example, surface cleaning, fixation, rinsing, dehydration, drying, mounting and coating, or embedding, cutting, and staining. Depending on the specimen to be investigated, these multi-step preparation techniques need to be carefully adapted in order to obtain electron microscopic art work that is artefact-free and useful for scientific interpretation. This paper describes the major risk factors and successful protocols that have been specifically adapted to the electron microscopic investigation of mollicutes.

2. The uniqueness of mollicutes as biological specimens for EM

The mollicutes are special microbes in many regards. They are the smallest known free-living and self-replicating microorganisms that are totally devoid of cell walls and intracytoplasmic membranes; they have only one type of membrane, the plasma membrane [8]. The mollicutes are incapable of synthesis of peptidoglycan and its precursors, and are consequently naturally resistant to penicillin and its analogues. However, they are quite sensitive to lysis by osmotic shock, detergents, and alcohols [9]. Thus, investigators using EM must be aware of the extreme fragility of these unusual microbes.

Mollicutes are believed to have arisen from Gram-positive bacteria with low genomic G+C contents (lactobacilli, bacilli, and streptococci lineages) and underwent a significant genome reduction (approximately 10% to 50%, depending on the genus) during evolution [10]. More specifically, these microbes lost cell wall synthesis genes, some biosynthesis genes, and some rRNA genes. Mollicutes have genome sizes ranging from approximately 500 kb to 1,700 kb. Mycoplasmologists believe that between 500 kb and 800 kb, perhaps even less, is the lower limit of genetic complexity needed by a self-replicating organism for life on Earth [10, 11]. Furthermore, it is believed that the small genome size and the associated limited number of protein coding genes is a major reason why these prokaryotes have adapted to a life in close proximity to their hosts. Many mollicutes are parasites, commensals, or saprophytes, and many are well described as pathogens of humans, animals, plants, and insects [9, 11-13]. The many unique properties of mollicutes that will be discussed in this section are summarized in Table 1.

Table 1 Properties of mollicutes compared to eubacteria, and their implications for EM investigation.

Property	Eubacteria	Mollicutes	Comments
Cell wall	Yes	No	The cells of mollicutes are extremely fragile
Genome size (kb)	> 1,500	~500 to 1,700	Mollicutes are “minimal prokaryotes;” many seem to grow best in close proximity to host cells
Sterol and fatty acid requirement	No	Yes, but not all (exceptions: e.g., acholeplasmas, asteroleplasmas)	Mollicutes require complex media; need to harvest at log phase of growth for best EM
Cell size (µm)	> 1.0	Most are in the range of 0.3 to 1.0; some are ~0.1 (e.g., ureaplasmas)	Need EM to view and characterize mollicutes
Terminal structure (attachment organelle)	Some have appendages (e.g., pili, fimbriae)	Terminal structures (only observed in a few species)	Can be used for identification by EM; if not observable or present, use immuno-EM techniques
Intracellular location	Many	Only a few	Consider the possibility that some mollicutes are located intracellular, extracellular, or both
Fried-egg colony morphology	Only L-forms	Yes	Has not been well studied on EM level; consider close mollicute-mollicute interactions

Most species of *Mollicutes* require sterols and fatty acids for growth [9, 14, 15]. Thus, complex and expensive media need to be prepared to grow these microorganisms in-vitro either for primary isolation from their natural sites, or for EM investigation using stock cultures of frozen mollicutes. Although the media for in-vitro growth of mollicutes are quite rich in nutrients, some strains may grow in the laboratory so poorly that cell-culture procedures may be an alternative to consider. Because of the poor growth of these microbes in artificial media, it is important to remember that cultures of mollicutes used for EM investigation should always be harvested in the logarithmic growth phase. Otherwise, the investigator will observe on electron micrographs only misformed, dying, or even lysed mollicute cells.

The lack of a rigid cell wall is one of the most striking features of mollicutes. Because of it, the cells are pleomorphic, varying from spherical or pear-shaped structures (0.3 µm to 0.8 µm in diameter) to branched or helical filaments (0.3 µm to 0.4 µm in thickness and of up to 40 µm in length) [16]. The small cell dimensions of these unusual organisms require that any investigator who has an interest in

studying the structural cell biology or the cytotoxicity and pathogenicity of these microbes should seriously consider to becoming proficient in EM techniques.

Some mollicutes have terminal structures (i.e., specialized attachment organelles) that are used during the interaction with host cells. For example, *Mycoplasma pneumoniae* has a tip structure, *M. mobile* a head-like structure, *M. pulmonis* a stalk structure, *M. gallisepticum* a bleb structure, *M. genitalium* a truncated-tip structure, and *M. penetrans* a tip-like-structure. [17, 18]. These unique surface structures can be used for the electron microscopic identification of these microbes. However, investigators should be cautious because some of these structures are not always observable or expressed, such as in some strains of *M. pulmonis* [19]. In order to distinguish mollicutes from eukaryotic cell particles (e.g., vesicles and cell debris released from host cells during infection), it is advisable to use immunolabelling techniques in conjunction with EM to properly identify mollicutes.

In recent years, mycoplasmologists observed that some mollicutes have the capability to parasitize within eukaryotic host cells that are not naturally phagocytic in nature. This was surprising since mycoplasmas were considered as typical extracellular parasites for almost 100 years. We know now that at least some mollicutes have evolved mechanisms for entering and residing within non-phagocytic host cells. Examples include *M. fermentans*, *M. penetrans*, *M. gallisepticum*, and *M. pneumoniae* [18, 20-22]. Electron microscopists should consider the possibility that, when examining biopsies or other clinical and research specimens suspected of mollicute infections, these microbes may be found either extracellular, intracellular, or both.

A final specialty of the mollicutes is that these microbes form on solid media colonies that have a characteristic fried-egg appearance. This colony morphology is typically observed only by LM but can be studied in more detail by EM. The organisms form these fried-egg colonies by growing denser and deeper into the agar in the center of the colony than in the periphery. In the earlier years of mycoplasmaology, there were heated discussions about the relationship between mycoplasmas and bacterial L-forms that also form fried-egg colonies on solid media. Yet, genetic analysis has since shown that there is no relationship between them and L-forms of eubacteria can be produced by partial or complete cell wall removal, while mollicutes do not revert back to cell-walled organisms after a cell-wall attacking antibiotic has been removed [11].

3. Scanning electron microscopy of mollicutes

Scanning Electron Microscopy (SEM) is a powerful method for the investigation of surface structures of mollicutes. This technique provides a large depth of field, which means, the area of the sample that can be viewed in focus at the same time is actually quite large [3]. SEM has also the advantage that the range of magnification is relatively wide allowing the investigator to easily focus in on an area of interest on a specimen that was initially scanned at a lower magnification. Furthermore, the three-dimensional appearing images may be more appealing to the human eye than the two-dimensional images obtained with a transmission electron microscope. Therefore, an investigator may find it easier to interpret SEM images. Finally, the number of steps involved for preparing specimens for SEM investigation is lower and thus the entire process is less time consuming than the preparation of samples for investigation with a transmission electron microscope. However, SEM specimen preparation harbours various risk factors that can easily distort the integrity and ultrastructure of the mollicutes.

The basic steps involved in SEM sample preparation include surface cleaning, stabilizing the sample with a fixative, rinsing, dehydrating, drying, mounting the specimen on a metal holder, and coating the sample with a layer of a material that is electrically conductive [1]. Because each of these steps are crucial and will affect the outcome of the study, they will all be described individually in more detail below.

3.1 Cleaning the surface of the specimen

The proper cleaning of the surface of the sample is important because the surface can contain a variety of unwanted deposits, such as dust, silt, and detritus, media components, or other contaminants, depending

on the source of the biological material and the experiment that may have been conducted prior to SEM specimen preparation. If these deposits are not removed prior to fixation, this material may get permanently fixed to the specimen surface and it will be almost impossible to remove later [1].

Robinson et al. [23] as well as Bozzola and Russell [1] suggested that the specimen should be quickly rinsed in a suitable buffered solution of the appropriate pH, temperature, and osmotic strength close to the milieu from which the specimen has been removed. Perhaps the best way to clean the surface of mollicutes is to carefully rinse them three times for 10 min in 0.1 M cacodylic acid buffer (pH 7.3) at room temperature.

3.2 Stabilizing the specimen

There are various ways of stabilizing a biological specimen. Stabilization is typically done with fixatives. Fixation can be achieved, for example, by perfusion and microinjection, immersions, or with vapours using various fixatives including aldehydes, osmium tetroxide, tannic acid, or thiocarbohydrazide [1, 7, 23, 24]. For mollicutes, a simple chemical fixation by immersing the specimen in a 1.5% glutaraldehyde solution prepared in 0.1 M cacodylic acid buffer (pH 7.3) and incubated at 4 °C overnight appears in most cases sufficient. The use of a postfixative (e.g., osmium) has been described to improve bulk conductivity of the specimen [7], but does not necessarily provide a better stabilization of mollicutes prepared for SEM.

3.3 Rinsing the specimen

After the fixation step, samples must be rinsed in order to remove the excess fixative. Perhaps the best protocol for mollicutes is to rinse the specimens in 0.1 M cacodylic acid buffer (pH 7.3), starting with one time for 10 min, and then three times for 20 min at 4 °C. Some of the samples can be stored in this EM buffer for several months because the buffer contains arsenic which inhibits the growth of unwanted microorganisms in the specimen container [23]. However, I strongly suggest changing the cacodylic acid buffer at least monthly if the samples are to be stored in this buffer for longer periods of time.

3.4 Dehydrating the specimen

The dehydration process of a biological sample needs to be done very carefully. It is typically performed with either a graded series of acetone or ethanol. The protocol that proved most suitable for dehydrating mollicutes for SEM includes the immersion of the specimens in 50% acetone for 5 min, 70% acetone for 10 min, 80% acetone for 10 min, 90% acetone for 15 min, and 100% acetone (dried with CaCl₂) twice for 20 min at 4 °C. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions [23].

3.5 Drying the specimen

The scanning electron microscope (like the transmission electron microscope) operates with a vacuum. Thus, the specimens must be dry or the sample will be destroyed in the electron microscope chamber. Many electron microscopists consider a procedure called the Critical Point Drying (CPD) as the gold standard for SEM specimen drying. I would recommend this procedure and have performed it numerous times using liquid carbon dioxide as the transitional fluid. Carbon dioxide is removed after its transition from the liquid to the gas phase at the critical point, and the specimen is dried without structural damage [24]. It is very important to exactly follow the instructions of the manufacturer of the CPD apparatus or the ultrastructure of the sample may be significantly altered.

In some of my experiments, I tried a specimen drying process called Simple Desiccation (SD). This technique is essentially a simple air-drying procedure after fixation, rinsing, and dehydration of the mollicutes. SD is not easy to do and there is the risk that specimens collapse, flatten, or shrink

uncontrollable under these conditions [1, 25]. Although SD is faster and cheaper, this method is like “walking on a tight rope.” For the EM novice investigating mollicutes, I would suggest the safer method of CPD.

3.6 Mounting the specimen

After the mollicutes have been cleaned, fixed, rinsed, dehydrated, and dried using an appropriate protocol such as the one outlined above, specimens must be mounted on a holder that can be inserted into the scanning electron microscope. Samples are typically mounted on metallic (aluminium) stubs using a double-sticky tape. It is important that the investigator first decides on the best orientation of the specimen on the mounting stub before attaching it. A re-orientation proves difficult and can result in significant damage to the sample.

3.7 Coating the specimen

The idea of coating the specimen is to increase its conductivity in the scanning electron microscope and to prevent the build-up of high voltage charges on the specimen by conducting the charge to ground [1]. Typically, specimens are coated with a thin layer of approximately 20 nm to 30 nm of a conductive metal (e.g., gold, gold-palladium, or platinum). For the coating of mollicutes, I have used gold and gold-palladium, and found both suitable. To guarantee best results (i.e., to achieve an even layer of metal coating over the sample), I recommend carefully following the instructions that come with the sputter-coater apparatus.

After all the steps described above have been performed, the investigator is ready to view the mollicutes in the scanning electron microscope. This is the moment when the mycoplasmologist will find out whether or not the multi-step sample preparation for SEM was successful. It is important to remember that each step has to be performed to perfection in order to achieve SEM images that can be interpreted without the influence of artefacts caused by specimen handling.

4. Transmission electron microscopy of mollicutes

Transmission Electron Microscopy (TEM) has the advantage over SEM that cellular structures of the specimen can be viewed at very high magnifications. However, TEM sample preparation for mollicutes is longer and more difficult than that for SEM and includes additional steps such as postfixation, the embedding of mollicutes in a resin, the sectioning of samples, and the staining of semithin and ultrathin sections. Bozzola and Russell [1] pointed out that perhaps the least forgiving of all the steps in TEM is the sample processing that occurs prior to sectioning. In other words, a poorly prepared specimen is useless to the investigator, whereas problems during the sectioning can be relatively easily fixed by simply cutting and staining more sections.

Specimen preparation of mollicutes for TEM includes eight major steps: Cleaning, primary fixation, rinsing, secondary fixation, dehydration, infiltration with a transitional solvent, infiltration with resin and embedding, and sectioning with staining. The first two steps are essentially the same as those described for SEM specimen preparation. These steps are therefore only briefly mentioned below.

4.1 Cleaning the surface of the specimen

As discussed earlier, the best way to clean the surface of mollicutes from contaminants is to carefully rinse them three times for 10 min in 0.1 M cacodylic acid buffer (pH 7.3) at room temperature.

4.2 Primary fixation of the specimen

Mollicutes can be chemically prefixed by immersing the specimens in a 1.5% glutaraldehyde solution prepared in 0.1 M cacodylic acid buffer (pH 7.3) and incubated at 4 °C overnight.

4.3 Rinsing of the specimen

In order to remove excess glutaraldehyde from the samples, the mollicutes should be subjected to a thorough but carefully conducted rinsing procedure. Specimens can be washed in 0.1 M cacodylic acid buffer (pH 7.3), starting with one time for 10 min, and then three times for 20 min at 4 °C.

4.4 Secondary fixation of the specimen

The process of fixation is crucial for biological specimens and has many purposes. Fixation can help preserving the structure of the specimen with no alterations from the living state [1]. When a biological experiment is completed prior to EM, there is always the risk that potentially destructive autolytic processes begin altering the sample before fixation takes effect. Fixation is also important for protecting the specimen during steps such as embedding, sectioning, and exposure to the TEM electron beam which operates at higher accelerating voltages. It is important to realize that no fixation procedure is ideal and that any type of fixation is likely to cause some artefacts on the specimen [1]. Minimizing the risks of artefact induction includes the selection of the most appropriate fixation protocol for a particular specimen.

Mollicutes can be successfully stabilized for TEM investigation by postfixation with 1% osmium tetroxide prepared in 0.1 M cacodylic acid buffer (pH 7.3) for 1.5 hrs at room temperature (immersion fixation).

4.5 Dehydrating the specimen

For TEM investigation, mollicutes can be dehydrated in a graded series of ethanol. More specifically, the following protocol is useful: Dehydration of mollicutes in 50% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min, and 99.9% ethanol (dried with a 4-mesh molecular sieve) twice for 20 min at room temperature. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions [23].

4.6 Infiltration of the specimen with a transitional solvent

The reason why this step is required is that the ethanol is not miscible with the plastic embedding medium I found most suitable for TEM investigation of mollicutes. The replacement of the dehydration solution by another intermediary solvent (i.e., propylene oxide) is thus necessary [1, 23, 26]. This process is essentially an alcohol substitution.

The immersion of mollicutes in propylene oxide twice for 20 min at room temperature is sufficient before attempting to embed the specimens in a resin.

4.7 Infiltration with resin and embedding the specimen

Mollicutes can be embedded in a variety of different media depending on the use (e.g., conventional TEM or immuno TEM). For conventional TEM of mollicutes, the epoxy resin Durcupan ACM is quite suitable.

The following protocol can be used: Immersion of mollicutes in propylene-oxide/Durcupan-ACM (1:1; v/v) at room temperature overnight (use gloves and a fume hood, and leave the specimen container open for the propylene oxide to evaporate). The next day, the specimens should be immersed in a freshly prepared Durcupan ACM mixture (pure) and left for 2 hrs at room temperature. A second Durcupan ACM mixture (pure) is then prepared and used as the embedding medium (free of air bubbles!).

Polymerization of the epoxy mixture can be achieved by placing the specimens in a drying cabinet for 2 days at 40 °C and for an additional 2 days at 60 °C. Leaving the samples after heat polymerization for an additional 1-2 weeks at room temperature can improve the subsequent cutting experience as the resin blocks continue to harden during this time.

4.8 Sectioning and staining of the specimen

The procedure for cutting specimens into semithin and ultrathin slices (sections) is known as microtomy and ultramicrotomy, respectively [1, 23]. Semithin sections (0.5 µm to 2 µm) were typically stained with toluidine blue for 1 min on a hot plate (70 °C to 90 °C), examined by LM, and used for identifying the specimen within the resin block before proceeding with ultramicrotomy. Ultrathin sections (about 70 nm to 90 nm) were typically stained with uranyl acetate followed by lead citrate.

As mentioned before, sample preparation for TEM involves more and some different steps than those for SEM. Like in any multi-step preparation procedure, virtually every step can affect the quality of the final electron micrograph. It is therefore important that the investigator plans and executes every step in great detail. I believe it became clear that these procedures involve a significant time commitment and require patience and skills that come only with practice.

It is important to mention that most of the chemicals used in EM are dangerous. Bozzola and Russell [1] wrote an excellent chapter on safety in the EM laboratory. They emphasized the importance of training in the proper use of all equipment and reagents in the EM laboratory. They also mentioned that the investigator must be aware of potential hazards such as fire, chemical, electrical, and physical associated with these items. EM facilities usually offer training and orientation programs that I highly recommend not only for the novice in EM (first learning) but also for the seasoned investigator (continuous learning).

5. Electron microscopy for studies of mollicute host-cell interactions

When specimens are used in interaction studies, special consideration needs to be made. These samples contain more than one type of organism; in addition to paying attention to the mollicutes, an investigator must also take special care of the host cells (e.g., respiratory epithelial cells). Both organisms must be given adequate treatments prior to EM (e.g., during the procedures for establishing explant cultures of respiratory epithelium and during the subsequent infection experiment) as well as during SEM and/or TEM specimen preparation. If one type of organism (the host cells or the mollicutes) is not optimally cared for, the electron microscopic data are essentially useless because they will most likely contain numerous artefacts.

Some of the factors that influence EM studies of the interaction between mollicutes and host cells include the following:

- Euthanasia procedures for laboratory animals needs to be carefully reviewed as some drugs can affect the function and structure of ciliated cells of respiratory epithelium
- The procedures for the establishment of cell cultures and explant cultures can influence the EM data (e.g., tracheal explants prepared with scalpels show on electron micrographs more damage than those prepared with razor blades)
- Media used for the cultivation of mollicutes and explant/cell cultures need to be carefully selected (a poorly maintained tracheal explant or mollicutes taken from poorly frozen stocks will most likely reveal significant cell damage on electron micrographs)
- Some eukaryotic cells (e.g., respiratory epithelial cells) have surface-exposed structures (e.g., cilia and microvilli) that can be easily damaged during handling; this damage may be later falsely attributed to the infection with mollicutes)
- Infection of some mollicutes on respiratory epithelium results in the accumulation of significant amounts of mucus and cell debris which requires a balanced washing protocol of the specimens to determine attachment characteristics of the mollicutes and cytopathology induced

Interaction studies between mollicutes and host cells have provided significant knowledge of the cytotoxic and pathogenic properties for many of these unusual microbes. The fact that mollicutes lack a rigid cell wall makes them an ideal study object for interaction phenomena because the interaction occurs directly between the plasma membrane of the mollicute and the plasma membrane of the epithelial cell. Thus, this model is perhaps the simplest of a pathogen host-cell interaction. Scanning and transmission electron micrographs of mollicutes interacting with respiratory epithelial cells are shown in Fig. 1.



Fig. 1 a) Scanning electron micrograph of respiratory tracheal epithelium in-vitro infected with *Mycoplasma mobile* strain 163 K. This mollicute attached to epithelial cells and caused significant damage (deciliation and exfoliation of epithelial cells). Magnification: 1,000x; accelerating voltage: 6.2 kV; sputter-coating: gold. b) Transmission electron micrograph of the attachment of *M. mobile* to epithelial cells. Magnification: 33,800x; accelerating voltage: 80 kV; ci: cilia; m: mycoplasma; mv: microvilli.

6. Conclusions and future outlook

The detailed structural examination of mollicutes as well as the characterization of the interaction between these microbes and host cells would not be possible without the electron microscope. Both SEM and TEM have proven over the years to be invaluable tools in this regard. Although SEM generates a different set of electron images than TEM, and thus provides an investigator with different scientific data, the combination of these two methods in a single investigation can be extremely powerful in that the findings generated by one technique often complement the data of the other (see Fig. 1).

This paper showed that EM is not an easy technique. There are many possibilities that an investigator may not get any usable electron micrographs for interpretation. The reason is that the preparation of specimens for SEM and TEM includes a series of steps; a single mistake in one of these steps will affect all remaining steps, and thus the outcome of the entire study. Furthermore, I discussed that laboratory experiments that were conducted on specimens prior to EM sample preparation can additionally influence the results. This “weakest link” issue of EM must be understood by scientists who want to use electron microscopic methods in their research. Despite the risk factors involved, SEM and TEM techniques provide fascinating images of biological specimens, in particular of the smallest free-living and self-replicating life forms on Earth – the mollicutes.

Finally, I wish that more mycoplasmatologists would enter the field of electron microscopic imaging as this would allow exchanging ideas and EM research protocols that are useful for the ultrastructural examination of these unusual microbes. There are many new recent advances in electron imaging technology providing numerous new tools for viewing and characterizing microbes. This should be

enough reason to attract young scientists who develop an interest in “playing” with these powerful machines and applying the generated data to the fields of mycoplasmaology and microbiology.

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