Chapter 6

Assessing the Viability and Degeneration of the Medically Important Filarial Nematodes

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Additional information is available at the end of the chapter

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Abstract

The assessment of nematodes as they generate and die is not a simple thing to do due in part to the complexity of the organism, and the fact that still relatively little is known about their physiology and internal biology. Indeed, the pathological changes in the internal organs of the worms are still only recognized in general terms. Obviously dead worms are easily recognized (when fractured, or calcified, etc.) but the lesser obvious changes can be difficult to detect and interpret. The point at which a worm can be defined as dead is not a simple matter; cessation of motility is currently the most commonly used parameter for this but it is not always a robust indicator and better indicators are needed. Various methods can be used to assess the presence, viability, and functionality of nematodes but these must be used with an understanding of the situation at hand and the specific questions being addressed. Careful use of appropriate statistics is essential given the complex nature of the target organism and the variability in the changes that can be seen within even one anatomical component of these worms. Histological assessment of the parasites present in both parasitized host tissues and isolated worms used in *in vitro* experiments can provide information that gives a more detailed understanding of the changes in nematodes as they degenerate and die. Understanding of the pathways nematodes follows as they degenerate naturally or under various external pressures, such as chemotherapy, remains a fascinating and potentially productive goal for investigation. Likewise, a complete understanding and definition of specific indicators that reflect parasite load, parasite viability, and damage, or reduced fecundity, will greatly help the fight against those nematode infections that currently cause significant burdens of disease in humans and animals.

Keywords: filarial nematodes, assessment, viability, death, histopathology

1. Introduction

Nematodes commonly infect humans, animals, and plants in all the ecosystems from the tropics to the polar regions; they can cause significant damage and consequently are responsible for some of the major chronic infections of these hosts. This being the case there is a great need to develop better treatment and prophylactic procedures to reduce the pathological effects of these infections, much of which are caused by events associated with the degeneration and death of the causative parasites in sensitive host tissues. Successful development of new effective and safe chemotherapeutic agents, a leading approach in controlling these detrimental effects, necessarily requires improved and more accurate assessment of the viability of these organisms. This is needed to both develop control mechanisms as well as to determine the epidemiological nature of these parasites.

In general terms, much is known about the effects of many parasitic nematodes on their human and animal hosts but comparatively little about the effects of the hosts, or chemotherapeutic agents, on the parasites themselves, i.e. the pathology of the parasites. There is, however increasing knowledge about model nematodes such as *Caenorhabditis elegans*, but still in comparison very little is known about the detailed biology and pathobiology of the more complex nematodes that commonly infect humans, animals, and plants; nor is it clear how useful it is to compare the model nematode, *C. elegans*, with the parasitic nematodes. A better understanding of these parasites in terms of their vital functions and their various pathophysiological changes - such as their mode of nutrition, the internal changes that lead irreversibly to their death, and the definition of specific parameters that indicate their viability - are essential to progress in this important field of research and development. Improved methods of controlling parasitic nematodes have the potential of improving the medical care of millions of humans and animals, as well greatly improving the yields of production crops.

Many examples exist in disease management suggest that it is important that we gain a clearer understanding of the biology of the infecting nematodes and the effects that the host and drugs induce in these parasites. Plant parasitic nematodes cause very significant problems to major crops throughout the world including vegetables, fruits and grain crops [1]. Some of the most devastating of chronic tropical diseases in human medicine are caused by nematodes; indeed, a major global health effort has been underway for some years aimed to control and eliminating a few of these tropical diseases. Two of the most successful programs today involve filarial nematodes, one that causes "river blindness" (onchocerciasis) and a second that is responsible for "tropical elephantiasis" (lymphatic filariasis). Well over 100 million people are affected by these parasites, and many more people still are at risk of these infections. The veterinary world has long understood the importance of parasitic infections, especially the persistent intestinal nematode parasites, with their ability to compromise growth and development of domestic animals. In both human and animal infections, the primary approach to reduce and eliminate these parasites has been for almost a century using chemotherapeutic agents - agents that either primarily damage and destroy the infecting organism, an event that can often induce a reactive pathological response in the host, especially with tissue based nematodes.

Central to measuring, understanding and treating parasitic infections in animals and humans are two fundamental parameters–worm load (i.e. number) and the worm viability; for many years, these have been assessed by active counting the number of viable worms (or a more easily detectable parasitic stage such as eggs, etc.) present in the host. Nematode infections in humans and animals that cause significant disease are essentially found in three major locations: in organs (e.g. digestive and respiratory tract lumens and ducts), in connective tissues, or in the circulatory vessels (lymphatics and blood vessels). Those parasites that lie in the gut are perhaps the most well-known and in many ways the most studied in human and animals, and are arguably more commonly seen because their cycles include detectable faecal stages. Thus, the time-honored test for assessing loads of these parasites has been the measurement of their egg production by the parasites (i.e. fecal egg counts through a variety of well-described methods: McMaster, Kato-Katz, mini-Flotac and other various egg concentrating methods) [2–5]. In recent years, there has been the gradual development and validation of molecular (PCR) approaches for estimating intestinal nematode presence and load, and it is likely that this type of technique will be used more commonly in the future.

In the case of *in vitro* experiments direct observations, such as motility, are used to distinguish between live and dead worms. This latter procedure, which appears at first glance to be relatively easy, is in fact not necessarily so, and thus there is a need to better understand the processes and indicators that are associated with the degeneration and death of parasitic nematodes. In more recent years other indicators of infection such as the presence of specific antibodies and circulating antigens derived from the worms have entered into the diagnostic menu; currently, an even wider range of indirect indicators of infection, such as parasite-derived microRNAs, are being investigated. In this present chapter, we will focus more specifically the issue of defining the viability of parasitic nematodes through direct means rather than the wider area of clinical diagnosis. We use as our model filarial nematodes which, are as described above important human and animal parasites, and where the major intervention used to control and eliminate these infections in medical terms is chemotherapy.

2. Filarial nematodes

Filariae are a very diverse group of nematodes that infect a very wide range of specific hosts. The three major filarial human nematode infections are river blindness or onchocerciasis (*Onchocerca volvulus*), lymphatic filariasis (*Wuchereria bancrofti* and *Brugia* sp.) and loiasis *(Loa loa*). In canines, *Dirofilaria sp.* are the most important filarial nematodes, and exists in most areas of the world. The filarial nematodes we will refer to in our discussion here focused on a morphological approach to assessing nematode viability are found naturally either in the major lymphatics and blood vessels (*Brugia* sp.), or in tissues and small vessels of connective tissues (*Onchocerca* sp.). The transmission these filarial parasites involves blood-sucking vectors, and the life spans comparatively long for the adult worms (5–12 years); their life cycles are depicted in summary in **Figure 1**.

Filarial worms are one of the best examples of a group of parasitic nematodes where a better understanding of the biological status of the parasite, or at least of certain parasitic stages, is

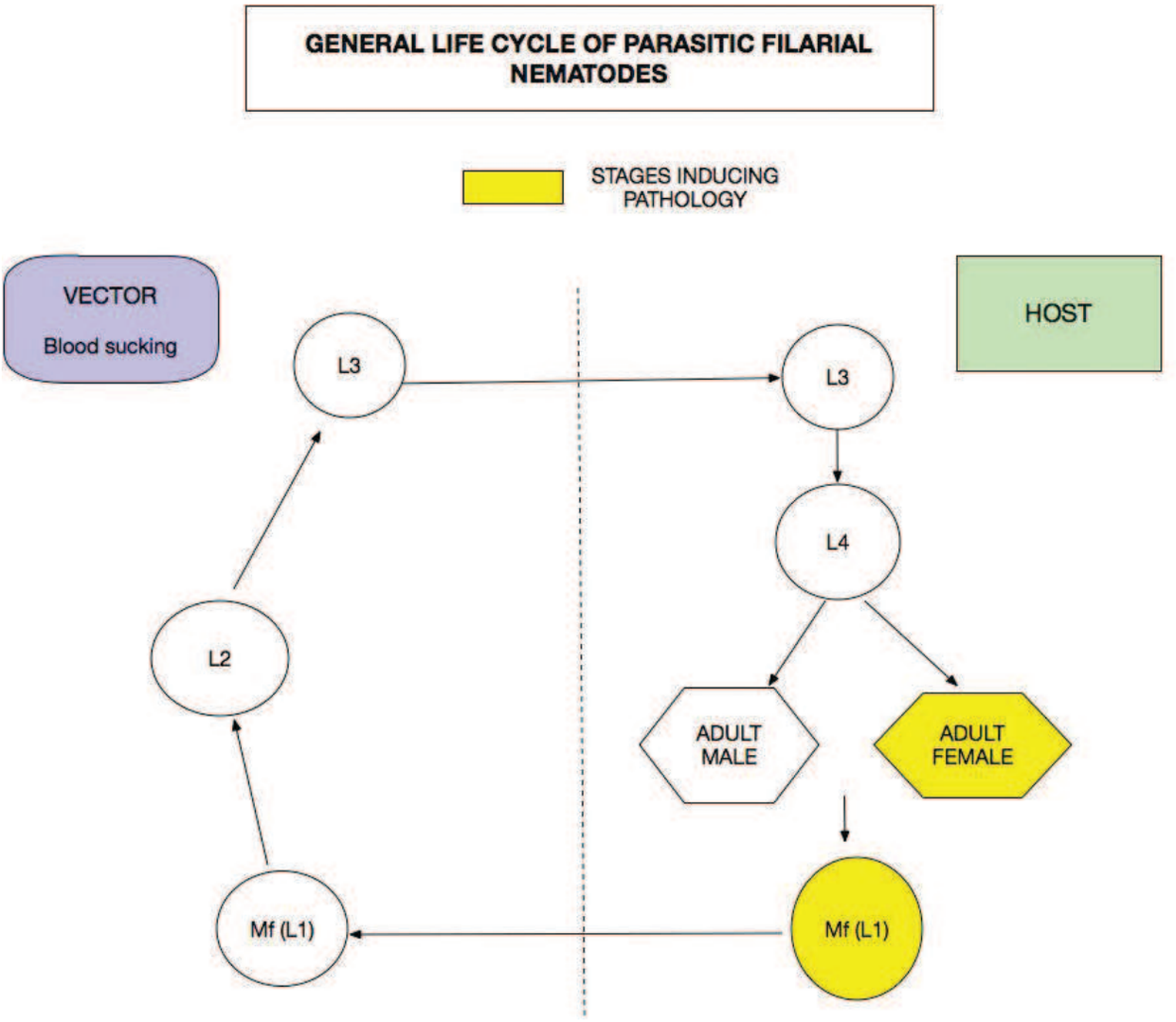

Figure 1. The general life cycle of parasitic filarial worms.

essential to establishing optimal effective and safe therapies, and to obtain a clearer understanding the pathogenesis of the disease in the host. Such an increased understanding is particularly important since these parasites infect and effect internal tissues such as connective tissues and circulatory vessels; this makes them more difficult targets for control compared to those nematodes that reside in the intestine where often treatment results in complete rejection of the damaged organism from the body.

3. Assessment of parasitic nematodes

In assessing a nematode infection in a host, it is important to select the most appropriate stage of these five-stage parasites to focus on and use as an indicator. This depends very much on the life-cycle in the host, as well as the availability of suitable techniques for assessment. For example, if one is trying to break infection transmission to a vector then the crucial stage that

provides the most important information in terms of epidemiological control is usually the stage entering the vector; in the filarial infections, this is almost exclusively the microfilarial stage. However, it is possible that permanent alterations in an earlier phase or stage may also be a strong and useful indicator that can predict termination of transmission. In our example here, evidence of a destroyed capability to reproduce and produce the first stage forms (the microfilariae) seen in significant uterine damage, and thus a lack of production of microfilariae, is an important indicator of the breaking of transmission. Observing such changes in adult female worms may indeed be more practically feasible than detecting the presence of transmissible microfilariae in the host or in the blood-feeding vectors. The effective break in the parasite's cycle, seen here in the permanent uterine damage and ceased reproductive ability, also shows that the actual death of the female worm is not the necessary target endpoint for defining a successful therapeutic agent or intervention: female worms may still be thought to be alive but have permanently lost the capability of reproducing. This also demonstrates the fact the simple counting of worm numbers often needs to be supported by an assessment of the internal anatomy of worms (e.g. the uteri or other vital structures) rather than just the number of whole worms present; a sterile worm is functionally as important as a dead worm. Thus, it is important to have methods of assessing the functional anatomy of worms. In both gut-residing nematodes and the tissue/vessel filariae, it is arguably more important to understand the functional state and reproductive capability of adult worms than just their physical, or numerical, presence. Measuring such parameters can either be done through histopathology examination as described below, or by the identification of products from components of the worms such as the uterus that may be released and be able to be detected in body fluids.

Assessment of the reproductive organs of male worms may also be a useful target for assessment; male filarial parasites are in the minority, and are probably fertilizing more than one female. Given that the reproductive cycle of filariae is comparatively long (months), it is likely that the female–the producer of the transmissible stage–is overall a better indicator of the host's infection status than is the male. The other stages that occur in hosts after infection, the third and fourth parasitic stages, are in general both hard to detect physically and exist for relatively short periods compared to the other stages; they have not been to date very useful targets for parasitological assessment. However, an indirect assessment of these stages through stage-specific antibodies or specific circulating antigens is becoming more feasible as the reagents for this type of test are improving rapidly.

It is important to re-emphasize, in the context of monitoring nematode infections, the distinction between using functional parameters and assessment that is through simple numerical quantification. In most cases a more detailed assessment that involves more functional parameters (biochemistry, fecundity etc.) is preferable. However, an exception where an estimation of the circulating load of the parasite is crucial and preferred is loiasis, caused by the filarial nematode *Loa loa.* This is a disease that appears to cause relatively little pathology except following the administration of ivermectin or diethylcarbamazine, anti-filarial drugs that are commonly used in treating filariasis. When these drugs are administered to individuals who carry high loads of circulating microfilariae their blood (>20,000 microfilariae/ml) severe reactions can occur and there is an increase of these people dying or being permanently affected due to vascular damage in their CNS tissues. It is, therefore, crucial that before treating with the two drugs mentioned

above the number of circulating parasites (the parasitic load) in their blood must be known. It is, in this case, not a matter of whether these parasites are functional (e.g. are able to be taken up by the vector and continue the life cycle), but what is the load of worms present and this done often by a standard blood smear estimation. The newly developed systems for measuring worm loads in blood improves the reproducibility and practicality when sampling in comparatively difficult situations such as in the field. Systems based on iPhone imaging technology (the LoaScope), and other utilizing light-scattering principles (the WiggleTron) [6], can now rapidly measure the number of parasites in blood smears at the field laboratory level.

The most common approach to assessing the viability of worms *in vitro* is through direct observation of their motion, commonly by visual means although image analysis systems have been developed. Motility has been used in numerous studies and has been the major approach used for the assessment chemotherapeutic agents for over 40 years. There are however the number of drawbacks at play using this approach including observer to observer variation, the lack of consistency of parasites' movement–often nematodes, including filariae, can be unable to move due to various local environmental reasons, such as inadequate culture fluid quality and sub-optimal temperature. However, arguably the most important challenge with using this technique is a lack of definition concerning the relationship between immotility and actual death of the worm. Techniques have been developed to improve the observation of motility in nematodes. For example, as the motion of most healthy nematodes follows a common repeatable pattern it is possible to detect alterations in these using detection systems that take many estimations of motion pattern over a short period of time. The "Wiggletron" system is one of these, a technique based on the recording of light deflected by the worms' motion, has improved the consistency of measuring worm motility *in vitro* and has been used to document the effects of various chemotherapeutic agents on filariae [6].

Deciding on the optimal means of assessing worm viability and number requires a consideration of the specific question being addressed, as well as the circumstances at hand. As mentioned above assessing nematodes *in vivo*, such as those present in tissues in or from infected individuals, usually requires a somewhat different approach from that need to investigate nematodes *in vitro*. A major challenge that must be addressed with studying this organism *in vitro* is that no robust culture system has been developed yet for filariae. Parasites used *in vitro* have inevitably come from an *in vivo* origin and may have already been affected by their status in their originating host, and are then placed in a compromised environment even before the test conditions are applied; this fact is often problematic in studies that look for degeneration and pathological changes in the worms. Understanding the genesis and form of subtle anatomic changes in such worms is therefore very important. Another complicating factor with filariae, both for *in vitro* or *in vivo* studies, is the fact that these worms are comparatively large and long. It is known that distinct degenerative changes can be in only one or two small sections of these long organisms and other areas in the same organ be quite normal, and thus it is relatively easy to fall foul of sampling error. Samples that statistically include a range of areas or sections in the worm are necessary for histological studies and the like. Similarly, motility–perhaps the currently most used parameter of "health" *in vitro* and indeed often used as a surrogate for the death of the nematodes–can be misleading with worms lying motionless to visual observation for long periods of time and then be seen to move again. More sensitive techniques that reveal,

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in a statistically robust manner, the type of motion that worms show over a longer time-period for example, do address this challenge to some extent. It is perhaps obvious to underscore here that in investigations of effects on filarial worms the parallel use of control samples is always essential, especially with *in vitro* experiments where the culture systems are less than perfect.

4. Technical aspects of assessing nematodes

The various techniques used to assess nematodes infections *in vivo* and worms that are maintained in vitro are summarized in **Figure 2**. The methods discussed here are focused on filarial worms, mainly because this is a parasite that has been the center of much of the recent research into better methods of controlling infections in humans. This is not to imply that there has also been a body of solid work in the same vein carried out in other disciplines, such as in plant pathology. The discussion here is focused on directly assessing worms themselves rather than the more indirect approaches through "footprint" surrogates such as antibody responses and circulating antigen.

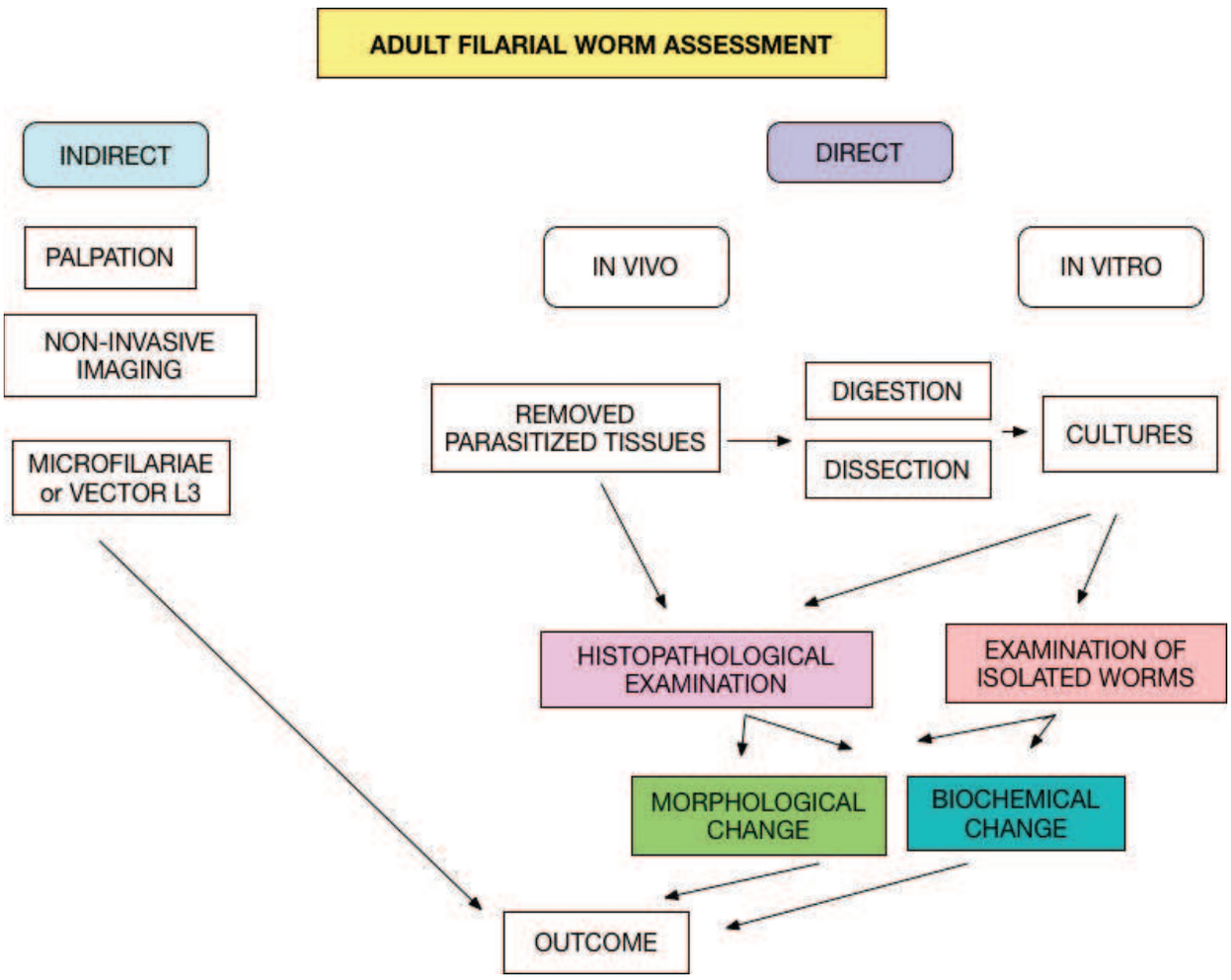

Figure 2. Assessing the status of filarial infections and filarial worms.

4.1. Indirect (clinical) assessment of population endemicity

Adult worms are central to the assessment the longevity of nematode infections, and this is particularly so in filarial infections, e.g. over five years with most filariae. The female adult worm is often used as the indicator worm rather than males because of its potential to produce large numbers of offspring and its remarkable longevity–arguably over 12 years in the case of *O. volvulus*. In assessing adult filarial worms *in vivo* there are several challenges. Although there is a site of anatomical predilection, in the case of LF it is the inguinal and femoral lymphatics, this is certainly not the only place these adult worms are found in the body. In onchocerciasis, the adults are found in "nodules" most commonly located in the pelvic girdle area (especially the iliac crest), but again can be found elsewhere such as on the chest wall and on the skull (the latter being more common in the children). *Onchocerca* nodules essentially are a nest of worms surrounded by fibrous inflammatory tissue. There have been very few autopsies carried out on individuals infected with this parasite but in the few that have been done these parasitized fibrous nodules have been found in the deep tissues along the femur bone, even when no externally palpable nodules are detected in the skin. Thus, although the presence of palpable nodules is arguably a good indicator at an epidemiological level it is not a particularly reliable indicator of individual infections. Currently, either the presence of microfilariae in the skin or eye, or a positive test to parasite-specific antibody remains the diagnostic tools of choice. With loiasis the adult worms can essentially be present anywhere on the body lying in the subcutaneous tissues and are known to migrate frequently under the skin, and in the external eye; in fact, loiasis is characteristically known for the fact that the adult worms can sometimes be seen migrating across the conjunctiva of the eye, hence the name "eye worm".

Therefore, manual palpation and clinical examination by experienced observers can be used to detect certain specific presentations of the main two filarial infections under discussion, with at least a moderate degree of reliability. The most reliable test being the assessment of the typical subcutaneous "nodules" (containing coiled adult worms and host inflammatory cells and tissues) in onchocerciasis; their presence in people living in a known endemic area has been to estimate the level of endemicity in the population of a defined geographic area. There are other causes of dermal nodules (e.g. dermal cysts, cysticercosis), but the typical location of onchocercal nodules on the body (iliac crest, the base of the spine, the chest wall or the head) increases the likelihood that such a mass is due to *O. volvulus.* The presence of nodules in adults has been used as an epidemiological indicator to catalyze the start of new chemotherapy control programs in endemic countries. Although it is highly likely that adult worms of this infection are also present in deeper tissues and therefore not able to be palpated, it is still likely that assessment of the prevalence of palpable nodules does reflect an acceptable degree a load of this parasite in a community or in an individual. In lymphatic filariasis swellings or lumps due to the presence of the adult parasite can be detected in the spermatic cord of infected males; these lumps (or 'nodules') are adult worm "nests": like onchocerciasis, these involve fibrous chronic inflammatory responses around the dead adult worms. These indirect reflections of infection are subject to misdiagnosis and are therefore often of limited diagnostic potential. However, their presence can greatly assist in interpreting any more general clinical signs of diseases that might be present in an individual; thus, the presence of typical nodules improves the diagnosis of onchocerciasis and lymphatic filariasis and the initiation of treatment.

4.2. Imaging of parasites *in vivo*

There are certain specific occasions in filarial infections where the adult worms can be seen by careful direct observation or using a diagnostic instrument. Loiasis has often been identified in people through the observation by the patient themselves of the migrating adult worm passing across the conjunctiva of the eye (thereby giving this parasite the common name "eye worm"). Questionnaire-based surveys using photographs of these worms active in the eye have been used with endemic populations to estimate the degree of endemicity of geographic area; such a frightening experience as watching a several centimeters long parasite pass across one's external eye–for example with women using a mirror to put on makeup in the morning–is dramatically memorable. Another example of the use of direct observation of the presence of worms is the identification by ophthalmologists using Slit Lamps to detect and count *Onchocerca* microfilariae present in the anterior chamber fluid of the human eye; in heavy infections almost 100 parasites can be present in this location–the observing of a 'Medusa's head" coiled mass of actively moving parasites in this location is not only dramatic and memorable but also provides a direct indication of the viability of the infecting parasites. Similarly using this ophthalmological instrument, or the simpler ophthalmoscopy, these microfilariae can also be seen lying within the cornea of the eye, often in association with small, usually whitish host reactions known as punctate keratitic spots; this is an example of where the death and degeneration of microfilariae can be directly observed and recorded.

Another non-invasive technique that has been used in both onchocerciasis and lymphatic filariasis to observe the movement of worms, and thus their presence and viability, is ultrasound imaging. Motile adult worms can be visualized in the lymphatic vessels of the inguinal canal of males infected with *W. bancrofti*, and likewise motile adult worms can be seen in the subcutaneous *O. volvulus* nodules–although the latter is less easy due to the worms here lying in tightly bound connective tissue rather than the intravascular location of the LF worms. Nevertheless, ultrasound is a technique that can assist both diagnosis and interpretation of the effects of chemotherapeutic interventions; however, it should be noted that this technique is relatively insensitive and best used as a supporting approach rather than the sole indicator in comparative studies. Recently developed techniques such OCT (optical coherence tomography) may be more sensitive.

4.3. Histopathological examination of infected host tissues

The histological assessment of parasite-containing tissues removed from patients is a very commonly used approach for assessing tissue (i.e. internally located) parasites, the subcutaneous nodules in onchocerciasis being a prime example in this present discussion. Most well-trained pathologists can identify the presence of a parasite in these tissues and do this by using some very simple characteristics that indicate the infecting organism (such as obvious outer walls containing non-mammalian cells), or by typical signs of a specific host reaction to these nematodes i.e. eosinophil and macrophage dominant host inflammatory responses. The viability status of the infecting nematode is usually only classified through major changes (e.g. its overall anatomical integrity, the breaking of the parasite wall, calcification etc.). More subtle changes in the parasite as it degenerates are still much less understood, therefore much less

described in regular histopathological reports of parasite-induced pathology. Nevertheless, such histopathological material is a vital window that can be used to describe the biology of the worm and its own pathology as it degenerates and dies. A good example is seen in advances in the understanding of the biology of many nematodes that has come in recent years from histological studies of the filarial endosymbiont *Wolbachia.* However, relatively little is described about other aspects of the changing anatomy in filarial worms as they degenerate, die and undergo pathological changes; nor is there much described with any of the parasitic nematodes. The careful examination of morphological changes within worms, either those *in situ* taken from infected hosts or those that have been exposed to drugs *in vitro,* is needed to develop a better understanding of the viability and state of degeneration of the worm, and will lead to more informative research findings, certainly more robust results than are produced from the simple recording of motility.

One of the characteristics of filarial nematodes that has inhibited studies at the histological level is the fact that these organisms are comparatively long and very narrow; the name "filarial" comes from the Latin word for "thread". There are many anatomical differences at different places along this almost 200–300 μm long adult worm. Indeed, it has been noted that the degree of change and degeneration in these worms can vary considerably from place to place within a single worm; this makes an assessment of developing degenerative changes in the worms hard to detect unless the worm has reached the stage of almost complete anatomical degeneration and the whole organism is changed. The more subtle changes that take place within this long organism as it progresses and degenerates towards obvious physical finality are poorly described to date. In fact, what change or changes that can be defined as the irreversible point(s) of degeneration of the worm remains unclear, and indeed may vary from species to species.

Many of the early morphological studies on the effects of drugs on filariae were focused on electron microscopic (EM) studies and although changes were seen using this technique, observations such as these (which are carried out at a very high power magnification) are notoriously poor for defining the overall changes in the observed worm or a group of worms. EM images usually only look at a very small proportion of the worm's complex anatomy, and thus do not give a good overall assessment of the status of the whole organism. Such detailed level techniques are however useful for defining specific anatomical characteristics such as those of the endosymbiont bacteria *Wolbachia pipiens;* electron microscopic studies and several immunochemical descriptions of these important organisms do exist in the literature.

A characteristic that has often being sought in filarial worms, and particularly with worms in adult filarial worm nests, is the age of the worm or worms being observed; such information with these long living worms is useful to those studying epidemiological questions. As these parasites ingest blood, and the products of its breakdown accumulate in the parasite's intestines over time, the presence of hemosiderin (often defined histochemically) in the gut has been used as an indicator of an older worm. Degeneration has been commonly defined by simple anatomical change although some histological markers, usually immunomarkers, have been used to reflect biochemical degeneration. One such example is the reduction in the molecule Nras, an important component in cell cycle maintenance, which occurs in adult female *O. volvulus* worms under the long-term pressure of *in vivo* chemotherapy with the anthelminthic ivermectin [7].

There is a major challenge in making statistically relevant observations on nematodes, organisms that have a lengthy anatomy and that often are coiled *in vivo*. Histopathological sections of onchocercal nodules naturally pass through several individual worms present in such coils and the identification of individual worms is not reliably feasible in most cases. It is statistically much more appropriate to regard each nest of worms as a single statistical entity, and assess the status of a whole nodule by observing and scoring the histological section as a whole; and in fact, to assess at least three 2–3 mm separated sections in a standard nodule of approximately 1 cm diameter; more slices may be needed depending on the parameter being assayed and the statistical power of the study in question. Various basic histochemical stains have been used with histological sections of parasite and the essentially routine stains, such as hematoxylin and eosin, together with stains that identifying certain chemical components such as carbohydrates, usually can provide substantial amounts of morphological evidence of change.

A caution must also be made here in that although nodules are usually removed by the surgeons as "single usually ovoid" structures, or collection of ovoid structures in heavy infections, and then these masses typically bisected through the longest axis for histopathological and other studies. These "halves" are not always equivalent in parasitic content; it has been noted that after long-term use of ivermectin the remaining adult worms often clustered in nests to one side of the ovoid nodule rather than being in the center as seen in untreated nodules. Thus, simple bisection does not necessarily provide equal content in these cases, and if one is bisecting a nodule to use two different assays to determining worm status–for example one-half for pathology and the other for molecular assay,–one cannot assume that the two halves are equal of similar in terms of parasitic content.

These basic principles in the preparation and assessment of onchocercal nodules (**Figure 3**) also apply to the examination of worm nests in lymphatic filariasis, although this is rarely done in human infections it is used in experimental models and in studies of other natural filarial infections where the worms are present in nests and nodules a such a *Onchocerca* sp. (e.g. *Onchocerca ochengi*) in cattle.

4.4. Isolation of worms from host tissues

In the case of onchocerciasis, the surgical removal of the fibrous subcutaneous nodules, and is in fact regarded as a therapeutic step, as it removes at least some of the fecund females; removal of nodules is also in some cases also a definitive diagnostic step. In many field situations, material collected this way also provides the opportunity for assessment of the viability of the worms in these nodules. This approach remains one of few readily available approaches to assessing the presence and status of worms *in vivo,* and for many studies it has also provided isolated adult worms for additional *in vitro* studies–the worms for these investigations being isolated either by careful manual dissection or by digestion of the fibrous host tissues using enzymes.

As these parasites are embedded in chronic host inflammatory responses, which in the case of filarial parasites usually contains a large amount of collagen, the general approach utilized for isolation is the use of enzymes to digest the worms free from the tissue. The most commonly

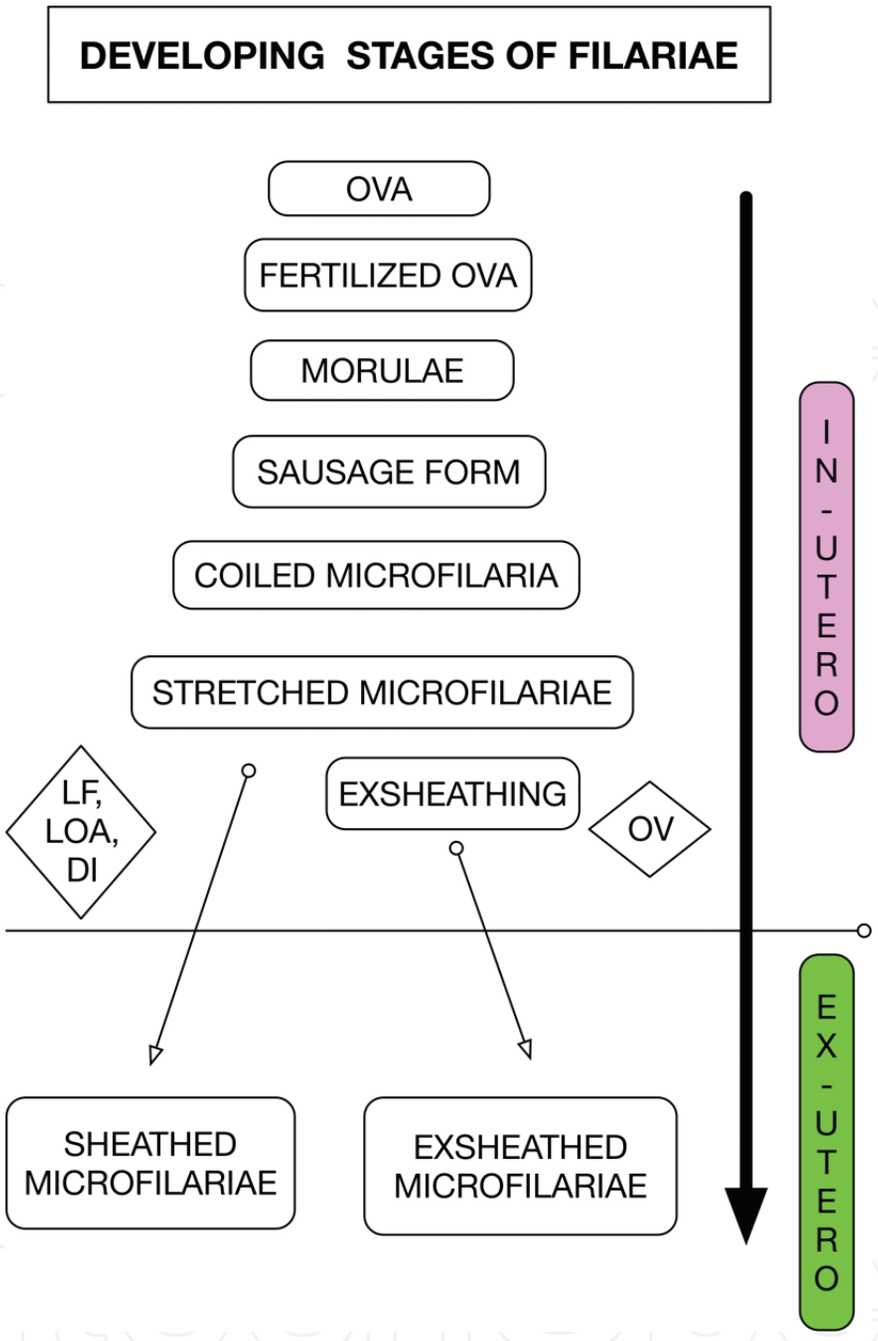

Figure 3. Methodology for preparing parasitized tissues and filarial worms for histological examination.

used enzyme is collagenase which targets the fibrous material [8]; the addition of dispase, a protease which targets fibronectin, collagen IV, and to a lesser extent collagen, is beneficial. It usually takes some 10–12 h to free the parasites in the case of *O. volvulus* nodules. This digestive process nevertheless can, and usually does, compromise many of the components of the worm themselves, although the major components such as the early uterine stages of microfilariae can be isolated from the digestates and easily counted. The re-implantation of cultured worms has also been used as an additional approach to testing viability [9, 10].

4.5. Assessing the changes in parasites

Alterations in worms that are extensively changed can often be seen by directly looking at the whole worm either *in situ* or *in vivo*. A typical sign of degeneration includes obvious breakage or decreased transparency (usually due to the degeneration of internal components and the accumulation of pathological constituents, e.g. calcification). In cases where there is severe damage with breakage or a marked increase density of the worms, it is likely that these worms are irreversibly damaged and this assessment requires only a simple examination with the naked eye. However, in some cases this visible degeneration is confined to only certain segments of the worm whereas other areas appear still viable and visually normal; care is therefore always needed in defining damage and the observing of the whole worm is essential (**Figure 4**).

It is the more subtle changes in nematodes that need better description and understanding of their significance. It is essential in this goal to understand the normal anatomy of the

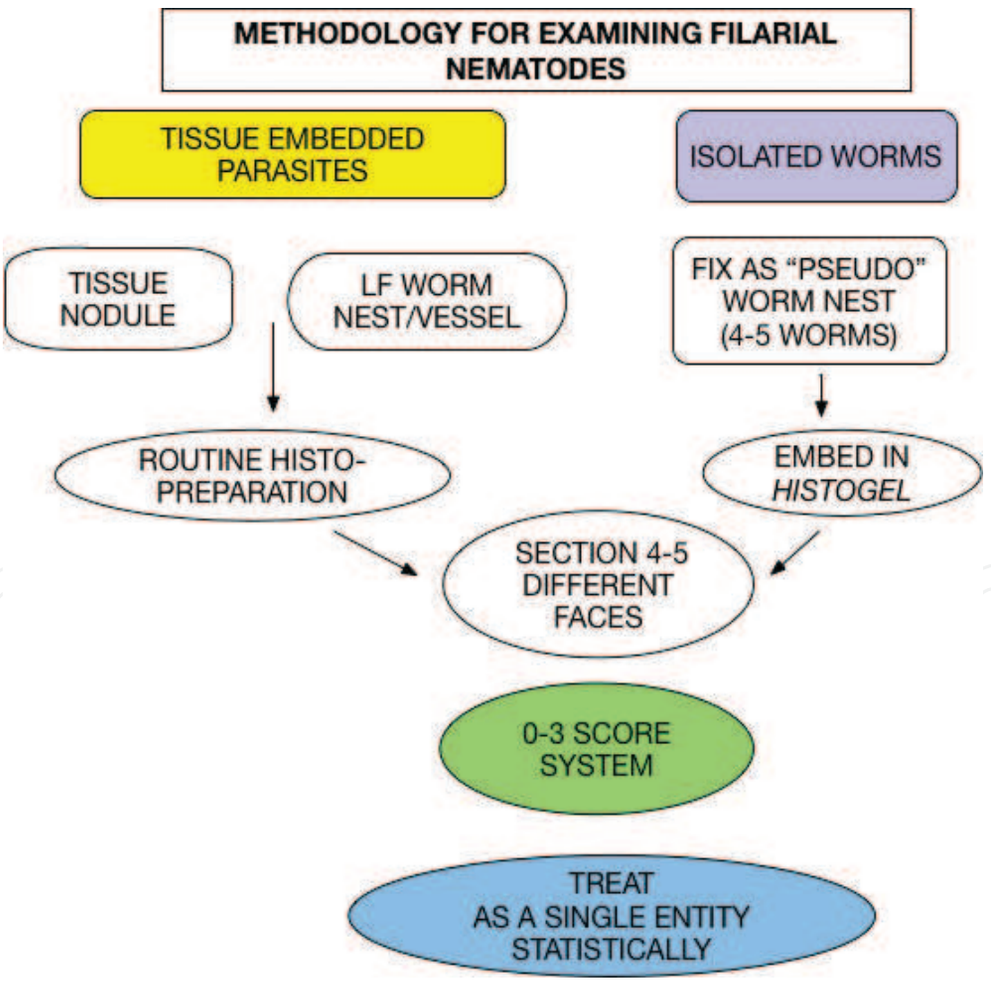

Figure 4. Stages of microfilariae developing in utero.

nematode before defining histologically changes that indicate degeneration and death. A summary of the major anatomical components that can be assessed is given in **Figure 3** and representative images in **Figure 5**. As already mentioned it is important to recognize that pathological changes within a degenerating worm can present differently at different points along the length of the organism, with changes occurring in one location and not in another within a single worm.

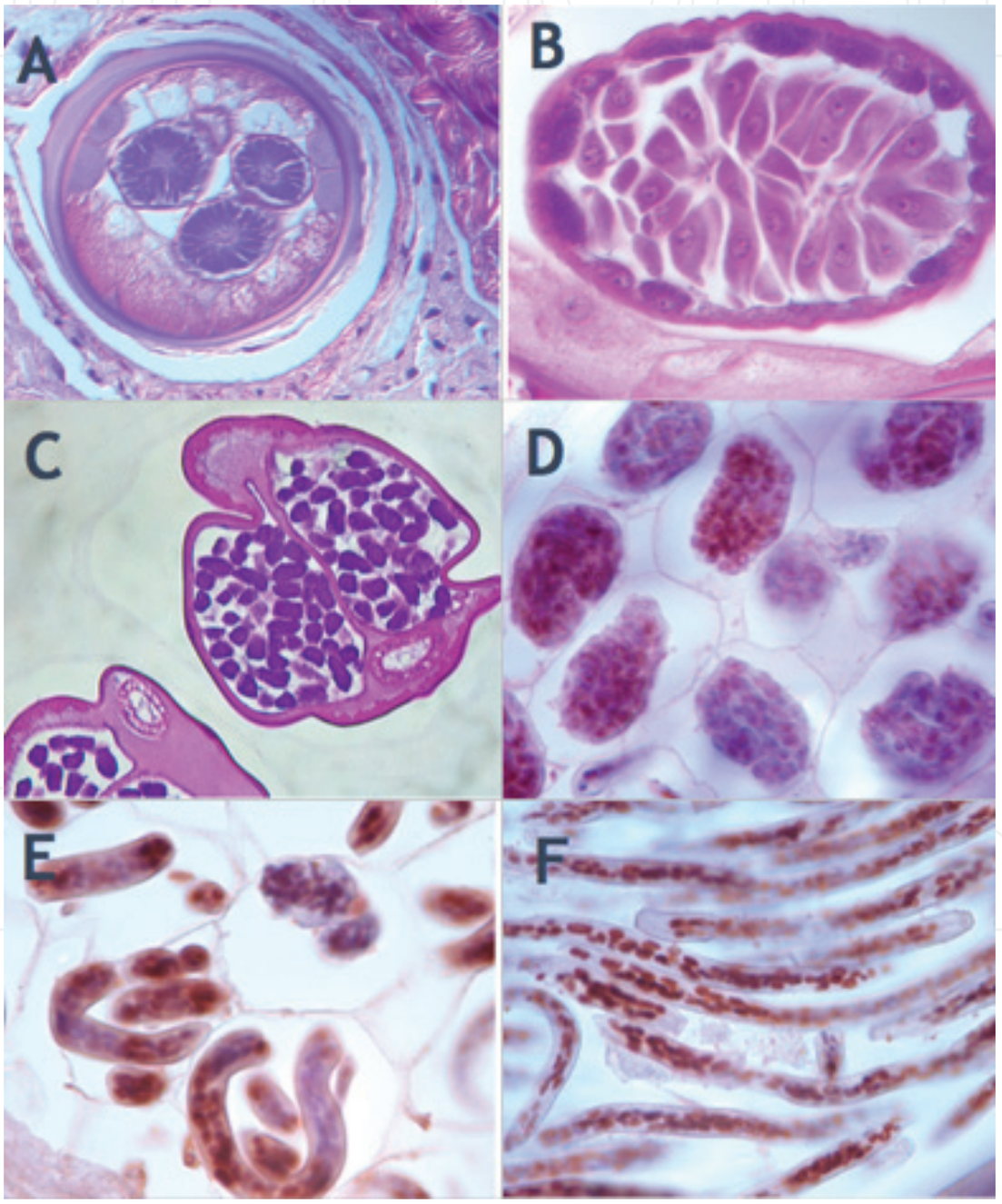

Figure 5. Normal components of adult female filarial nematodes. A. Ovaries of an onchocercal worm, B. Earliest stage of filarial ova, C. Morulae stages filling two uterine horns of a healthy female filarial worm, D. Mature morulae inside individual egg shells, E. Coiled microfilariae contained within egg shells, F. Fully stretched microfilariae ready for release from the uterus.

Different ways of assessing the state of worms in histological sections have been used. A scoring system that has been developed and used successfully for the investigation of new chemotherapeutic agents [11, 12] and the type of degenerating forms seen in such studies shown in **Figure 6**. The use of a four-level score (0–3) for subjective assessments has been generally accepted in the realm of anatomical pathologists as minimizing, as much as possible, differences between observers and is a suitable formula for assessing nematodes.

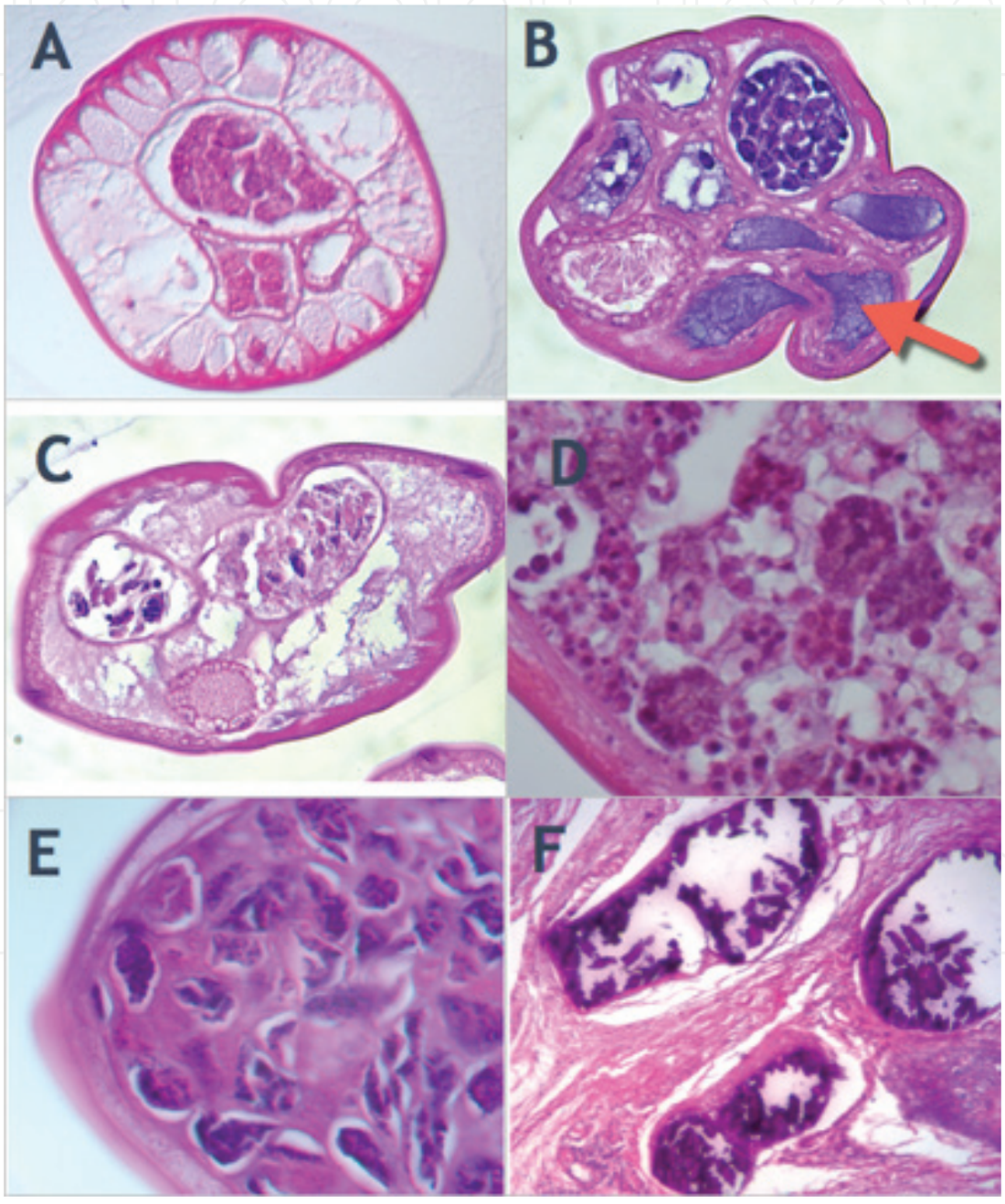

Figure 6. Examples of typical degenerative components of adult female filarial nematodes. A. Filarial nematode with damage to the body wall. B. Filarial nematode with damaged horns of the uterus with early calcification (blue staining of the uterine content), C. A degenerating worm with the morula stages unable to form. D. Disrupting early morulae forms E. Uterine horn of a damaged filarial worm containing many degenerating forms, F. Calcified casts of a dead filarial worm embedding in a chronic tissue response (nodule).

4.6. The assessment of *in vitro* worms

Much of what has been described for assessing parasites in *in vivo* situations applies to those parasites that have been obtained from culture systems. Here it is important to acknowledge that worms maintained *in vitro* are already in an unnatural environment and this can affect certain anatomical components more than others. The wall and cuticle of cultured worms often, even in the control samples, can have degenerative changes that are induced by this unusual environment. If worms have been isolated using tissue digestive methods before culturing then the changes due to the processing are consequently even more common.

A useful approach to preparing *in vitro* cultured worms for histology is to essentially prepare the worms as "nests" mimicking the natural situation seen in O*nchocerca* nodules, i.e. wrap the worms into a small artificial "balls", fix, embed and assess them as described above for the natural "nests" in onchocercal nodules (**Figure 3**). This collection of worms, optimally a minimum of 5 worms, is then regarded statistically as one entity (as are in vivo nodules). In cases where there is only a single worm available for a particular assay, then again, this worm should be coiled up into a small "ball" for processing; this approach allows for better statistical evaluation.

4.7. *In situ* markers of viability

The use of *in situ* markers is an important new approach being developed for assessing degenerative changes in nematodes but to date, there are still relatively few studies that address this issue in any great depth. One that has nevertheless been extensively described is the continuing presence of the required endosymbiont *Wolbachia*–usually identified by using labeled antibody markers against a primary antigen (WSP) of this bacterium. The presence of these organisms has been used as an indicator of the viability of the adult filarial worm. It must be recognized that this endosymbiont is not uniformly distributed along the worm and it is therefore relatively easy to be misled by only observing relatively few histological sections of the worm, many of which may naturally not actually contain this organism. It is also has been shown that MMP-2 and MMP-9 are two collagenases that are associated with *Wolbachia* in filariae, and a reduction in these two enzymes may reflect early damage to the adult worms [13].

There are several studies that have described several enzymes that appear to be present in filarial worms [14], and it is likely that specific enzymes will be identified soon whose presence could act as reliable indicators of worm viability. Biochemical approaches have been used to assess the viability of *in vitro* worms for many years–the formazan assay being commonly used [15–17]. Enzymes involved in general biochemical maintenance of nematodes [18], such as Nras have already been seen to provide some information as to the adult worms' integrity after chemotherapy [7].

4.8. Indirect markers

Although it is not a major purview of this discussion here to go into the wider area of laboratory and rapid test systems, it is nevertheless important to note that there is a considerable amount of experience over many years with the use of immunological markers, such as circulating antigens

and host antibody responses for the diagnosis and epidemiological assessment of filarial infections, both in humans, dogs, and other animals [19]. In fact, with human lymphatic filariasis, the major diagnostic tool used in major public health control programs is a rapid diagnostic test for detecting circulating antigen in finger-prick sampled blood. In human onchocerciasis, the current approach is to use the presence of parasite-specific antibody (Ov16) to indicate the status of infection in an endemic community. The use of samples of urine and saliva for these assays has been attempted but with varying and unfortunately rather unuseful results to date.

There is an ever-increasing number of studies considering whether or not circulating specific products of parasites (e.g. protein microRNAs, etc) can reflect both the presence infection (in terms of the presence of different parasitic stages) or perhaps the load of infection (the intensity of infection). This is an area of research that is vital to the efforts to eliminate the major parasitic diseases across the world. It is likely that in the next few years specific circulating markers will be identified in blood, or hopefully (for ease of collection) in urine or in saliva. This would provide a more practical way to assesses populations in epidemiological studies and lead us more quickly to the global goal of eliminating nematodes for affected populations.

5. Discussion

There are still many aspects of measuring the viability of nematodes that need improving. A major challenge is to determine when a population of worms that have been subjected to an intervention, e.g. chemotherapy, an immune response, etc., and are on an irreversible pathway to death, and thus the parasite no longer can contribute to the infection in question. To achieve this, it is necessary at the level of the worm itself to understand what are the actual changes, or pathological events, within the worm's anatomy and biology that reflect permanent irreversible damage.

As described above it is relatively easy to detect alterations if they are physically obvious (e.g. calcified, broken and obviously damaged entities) but it is the interpretation of the less obvious changes in worms or the deceased levels of a marker indicator that is difficult; what level of damage is irreversible? Optimally it would be extremely useful to define a single change or a simple collection of changes that reflect permanent irreversible damage. In the case of filariae it is likely that the uterus (the biggest organ in the female) is a useful indicator site for detecting damage and defining permanent damage. The body wall is also an important target organ for this role but it is an organ that is easily artificially altered by many of the isolation techniques used in preparing the worm samples for studies, especially those for *in vitro* studies. Another reason for focusing on the uterus is that interruption of the reproductive capability is a major intervention goal for the three major filarial infections of humans and would be extremely valuable to the success of the current global elimination programs for these two infections.

The assessment approach used needs to be driven by the question being asked when deciding on the method and focus of any assessment. The complexity of the life cycle of nematodes necessitates carefully focusing investigations on stages or anatomical components that are

likely to prove useful and provide practical information. The approaches used for assessment need in most cases to be closely associated with the programmatic question being addressed, and take into consideration the environmental situation at play; for example, the breaking of infection transmission or understanding the direct effect of a chemotherapeutic agent on the worm's reproductive capability. It is also important to distinguish between estimating parasite loads in an infected host and the measuring of direct effects on a parasite stage; although these two questions may be intimately linked they are not necessarily the same nor necessarily use the same method for assessment.

An obvious area of basic research that would greatly enhance the needs for developing better techniques for assessing parasite viability is advancing the knowledge of the basic physiological, biochemical and functional characteristics of nematodes and any species differences. Using *C. elegans* as the type model [20] is useful but filarial worms and other parasitic nematodes are considerably more complex and are likely to have different and unique characteristics. This kind of research is difficult to maintain in the present world where research funding is difficult to acquire, but nevertheless, the acquisition of more detailed information in this subject would undoubtedly be highly valuable. There is a tendency to interpret cell death, tissue damage and other pathological processes from the perspective of what we know about these processes in mammalian organisms and it would be extremely useful to know if there are similar or different processes occurring in metazoans.

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