Understanding and managing the gut ecology of Australian wildlife by light-microscopy diagnosis of faeces

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ABSTRACT: Wildlife rehabilitation organizations and off-site carers who provide treatment for endangered, sick, injured and orphaned wildlife, confront a number of gastrointestinal abnormalities which can be diagnosed by simple light microscopy of unstained faecal wet-mounts, or gram-stained dry mounts. Recognition of microbes in diverse wildlife species is based on morphological similarities to microbes that are important in human and veterinary medicine. Microbial pathogens must be recognised amongst a faecal background that varies according to wildlife species, diet and time of year. Faecal analysis is correlated with clinical symptoms to assist in proper diagnosis, quarantine, treatment and determination of outcome. Photographic documentation improves subsequent diagnostic ability and assists in teaching and research. The Kanyana Microscopy Station is working to achieve these goals. The conference presentation will show examples of easily diagnosed worm and protozoan parasites, and yeast and bacterial infections, which are commonly found amongst wildlife admissions at the Kanyana hospital. The case history of a Western Gray joey that died of a gut infection will be reviewed. Lessons learned from this case have important implications for pouch design, diet, and antibiotic and probiotic therapies, in the rearing of joeys for release into the wild.

I. Background

During the industrial revolution (1760-1830), humans migrated away from rural areas where they organically farmed and raised livestock, to live in crowded cities where they could find work. On the farms, health-care needs were fulfilled by traveling doctors and midwives, and infectious diseases were not a serious problem. Most farm women breast-fed their children and prepared homegrown food for the family, while men worked the land and tended to livestock. Fresh drinking water came from the ground or the sky. Livestock manure was composted and used to fertilise vegetables, which were usually eaten unwashed and uncooked. Human faeces was deposited away from the home, or contained in an outhouse. Farmers preserved foods by developing anti-microbial processes such as cooking, drying, salting, sugaring, pickling, canning, and refrigeration. They depended on microbes to make bread, beer, wine, vinegar, cheeses and yoghurts, not only for flavour but for food preservation. They drank beer containing yeasts, ate soured milk (yoghurts and cheeses), and consumed wormy fruits and vegetables, rather than waste food. Thus, farmers managed their microbial environment naturally, without the aid of a microscope, and were relatively healthy.

Following the industrial revolution, rural areas were taken over by mechanised agriculture, which permitted the over-planting of food crops using chemical fertilisers, and the housing of livestock at increased densities through over-production of animal feed. The crowding of livestock led to an increase in animal infectious diseases. Livestock faeces contaminated food destined for human consumption, providing a route for the spread of infections from animals to humans. In the cities, humans lived in crowded conditions with their animals, and untreated human sewage flowed into the same rivers that provided their drinking water. Their living quarters were infested with fleas, lice, roaches, flies and mosquitos, which carried microbes from one human to another. Humans contracted microbial diseases they had never before encountered, and masses of people died. Most of the deaths were due to the infection of mothers at childbirth, wound infections (tetanus, gas gangrene), pneumonia (viral and/or bacterial), sepsis (bacteria in the bloodstream), and severe uncontrollable diarrhoea caused by

bacteria or protozoans. Prolonged diarrhoea resulted in malnutrition, intestinal damage, and loss of vital body fluids (dehydration). Diseased people either died at home or were taken to hospitals, where they often died from hospital-acquired (nosocomial) infections. Maternity wards and surgical theatres were located near infectious disease wards and morgues. Hospital hygiene was nonexistent. At one point, the chance of dying from a nosocomial infection was greater than the chance from doing nothing.

Before the 19th century, physicians did not know that infections were caused by microbes. Our knowledge of microbes was revolutionised by several important discoveries. Invention of the compound microscope by Robert Hooke in 1665 allowed humans to view bacteria, yeasts and protozoans for the first time. However, it would be 200 years later before the connection between infectious diseases and microbes (the germ theory) was scientifically proven by Louis Pasteur (1861) and Robert Koch (1876). Practices that we take for granted today, such as the washing of hands first instituted by Ignaz Semmelweis (1840), and the use of aseptic conditions during surgery established by Joseph Lister (1867). Novel vaccines were developed, in some cases without identifying the microbial source. The first vaccine was made against smallpox by Edward Jenner, at the end of the 18th century. Jenner noticed that milkmaids on the farm rarely developed smallpox, which was ravaging the cities, although they did develop a mild case of cowpox from the cows they milked. He reasoned that infecting people with cowpox could prevent them from contracting smallpox, even without knowing that it is caused by a virus. Thus, not all transfer of germs from animals to people are bad. Since the mid-19th century, the field of microbiology has been driven by a search for the germs which cause human and livestock diseases. Medical and veterinary textbooks are full of information about these 'bad guys'. However, pathogenic microbes represent only a small fraction of the total microbes in our environment, most of which serve a neutral or beneficial purpose (the 'good guys'). We know far less about these.

Whilst validation of the germ theory enabled humans to target disease-causing pathogens, it also caused humans to become fearful of their microbial environment (microphobia). New antibiotics and pharmaceuticals appeared to give humans absolute power over microbes. Early antibiotics were chemical antiseptics that could only be used on open wounds. Few options were available to combat internal infections, or to treat deep gangrenous wounds such as those seen in war casualties. Following the worldwide influenza virus epidemic of 1917-1918, which killed 21 million people mainly from secondary bacterial pneumonia, scientists began to search desperately for 'a magic bullet' to cure infectious diseases. Alexander Fleming discovered penicillin (1928) quite by accident, as he noticed that a fungus growing on his culture of pneumonia-causing bacteria was producing something toxic to bacterial growth. The subsequent mass production of penicillin opened a new door to antibiotic development, just in time for World War II, where it saved countless loss of limb and life. Most antibiotics are derived from fungi or soil bacteria which produce toxins and inhibitors against their main competitors, other microbes. However, four years after penicillin went into broad use, penicillin-resistant bacterial diseases began to appear, just as Fleming had warned. As more potent antibiotics were developed, pathogenic microbes mutated and acquired drug resistance in antibiotic-treated individuals. Each time chemists modified antibiotics to counteract the mutations, another round of selection for drug-resistant bacteria began. Also, because precise identification of bacteria can take days or weeks, and doctors wished to provide immediate treatment, broad-spectrum antibiotics became a remedy of first choice even when bacteria were not causing the disease.

Since validation of the germ theory, Western civilisation has gone to great measures to kill all germs, both good and bad. Our living environment has been disinfected and sanitised in the name of hygiene. Antibacterials have been added to detergents, soaps, deodorants and lotions. Commercials appear on TV urging people not to touch a ball that has been in a dog's mouth, and to use anti-bacterial lotions after doing so. Animals meant for human consumption are given prophylactic antibiotics in their feed to promote better growth. Preservatives are used to give foods a longer shelf life, but these chemicals may inhibit intestinal microbes in the way they prevent spoilage. Artificial sweeteners which cannot be metabolised by mammals may serve as food for microbial fermentation. However, these preservatives, sweetners, disinfectants, antibacterials, and broad-spectrum antibiotics have altered our personal microbial ecology, similar to the way that pesticides have altered the ecology of our land. Both 'bad guys' and 'good guys' are killed, upsetting the healthy natural balance, and opening the door to opportunistic infestations by drug-resistant organisms. The indiscriminate and widespread use of antibiotics in human and veterinary medicine has allowed microbes to evolve that today are resistant to all known drugs. The main causes of drug resistance are the use of antibiotics which are too dilute or have expired (too weak), and failure to complete the full course (too short).

As with wildlife ecology, microbial ecology is determined by interdependent cooperation and competition among species for food and habitat. Some microbes need a diet rich in simple carbohydrates (sugars), while others prefer complex carbohydrates such as plant fibre (cellulose). Microbes that can digest fibre are able to break down (ferment) complex carbohydrates into simple ones, which other microbes may use. Some microbes need amino acids in their diet to make proteins, while others can fix free nitrogen from the atmosphere or ammonia, and synthesize their own amino acids and proteins. Microbes that can fix free nitrogen may become food for those which cannot. Some microbes (aerobic) require oxygen for survival, while others (obligate anaerobes) die immediately in its presence, and some (facultative anaerobes) can live with or without it. When aerobic microbes consume all the oxygen in their environment (overgrowth), they die and begin to decompose. This sets up conditions favourable to anaerobic growth. Anaerobes produce gases and bad smells (putrifaction), and are associated with stagnant (non-aerated) decomposition. Some microbes die off when conditions become harsh, whilst others form spores that can resist harsh environments for decades, and generate new life when conditions become favourable again. Some microbes 'detoxify' their environment and allow other microbes to grow, while some produce toxins that inhibit the growth of other microbes (eg, antibiotics, antimycotics). Some toxins are secreted into the microbial environment (exotoxins), while some are part of the microbe itself (endotoxins). Endotoxin containing bacteria, such as *Salmonella*, may be normal for one species (eg, lizzards), but cause severe diarrhoea and shock in another (eg, humans).

Exotoxins and endotoxins are the cause of most microbial-associated diseases, whether from accidental ingestion (eg, food poisoning, botulism) or active microbial infections. Surprisingly, a number of toxic microbes may coexist harmlessly at low levels in normal microbial flora, as they are held in check by other microbes. Not surprisingly, conditions which upset this normal balance can favour the loss of beneficial microbes, and allow takeover by toxic ones. Some toxins produced by gut microbes (enterotoxins) may cause a bad case of diarrhoea but are not life threatening, while others may produce severe fluid loss leading to dehydration and death. Bloody or uncontrollable diarrhoea is a cause for immediate concern. Transient diarrhoea, which is controllable but may be loose/watery, is quite common in all species and can be caused by a number of factors. It may be associated with dietary changes, such as those occurring when weanlings begin to eat on their own, or seasonal changes in food supply. From the wildlife rehabilitator's perspective, it may be associated with changes from native to captive

diet, improper feeding, and even the stress of captivity itself. Sometimes, good-intentioned humans want to do everything in their power to save these creatures, but create more stress for the captive animal. Stress should be minimised as much as possible. Sometimes, patience and nonintervention are the best treatments. Regardless of the cause, transient diarrhoea that persists for more than a few days should be watched closely. Faecal diagnosis is an important tool in this regard.

At birth, all vertebrates have a sterile gut. Microbes start to colonise the gut when the newborn begins to take food. These microbes originate from a number of sources. For birds and reptiles, eggs are usually laid in a nest which is exposed to the environment. The eggs originate from the female cloaca, a common opening for both the intestinal and genital-urinary tracts, and their shells are covered with microbes. Also, the nests are full of microbes associated with parental faeces and feathers. Parents feeding their young transmit microbes present on the food already, or added during the process of regurgitation. In mammals, the newborn suckles a nipple that is covered with microbes, and receives milk through mammary ducts that contain benign microbial flora (similar to those used to make yoghurt). Changes in maternal milk composition, as the offspring develops, is associated with corresponding changes in microbial flora that prefer one formula over another. As a young mammal begins to eat solids, microbes on the food or in soil are ingested. The parents and young groom themselves and each other, a major route of microbial transmission from the anus to the mouth. Coprophagia (faeces-eating) in herbivores primarily acts to conserve nutrients that would have been lost from incomplete digestion of plant material, and is a common way that intestinal microbes (especially anaerobes) are transmitted from adults to their young. When baby koalas emerge from the pouch, they first feed on pap, a soft fluid excreted from the mother's anus, which serves as a rich food source loaded with intestinal flora. This marks the onset of accelerated growth.

As modern civilised humans, we may be repulsed by the thought of ingesting faecal germs, which relates to the above points. We may generally agree that it is natural for animals to live that way, but believe that humans should be different. Actually, humans today may be living too cleanly, for we have fingernails and were meant to eat dirt! This is the way that healthy animals in the wild behave, and it was the way of our ancestors until only recently in human history. Researchers in the field of gastroenterology are showing that in many cases, inflammatory bowel diseases may be due to unnatural balances in the way that microbes are interacting with each other, and with our immune system. Many human allergies to food and environmental antigens may be caused at least in part by a lack of exposure to harmless substances during pre-pubescent development, so we inappropriately respond to them during adulthood as if they are foreign invaders. Some allergies may also be caused by a loss of beneficial bacteria that would detoxify naturally allergic substances.

Even if we cannot understand the cause of bowel disorders, how do we reverse the imbalance? Current therapies are to give stronger antibiotics for the bowel infection, and/or anti-inflammatory drugs to quell the immune system. One new experimental probiotic therapy is to ingest soil nematodes (microscopic worms) that are not parasitic or toxic, which can reduce bacterial inflammation. The eating of harmless soil nematodes is something 'primitive' humans did quite commonly during our evolution. Research has shown that immunity to parasitic worms (and worms in general) can shift the response away from bacteria in the gut, and quell the immune system naturally. Similar studies are being conducted using yeasts and fungi. Other experimental therapies include 'stool transplantation' of healthy gut flora into individuals with bowel diseases, and the use of probiotic capsules and powders, which contain 'sanitary' beneficial bacteria. One of the best probiotics for mammals is naturally fermented ABC yoghurt, for reasons explained in Section III.

II. The Kanyana Microscopy Station

In the diagnosis of faecal samples as a way to monitor health of the animal, and as an indicator for treatment strategy, all of the concepts above need to be considered. To understand these concepts, it helps if one has formal microbiology training. However, self-teaching is feasible for most biologists and nurses, using cheap ex-college textbooks and the web. Kanyana plans to organize a practical microbiology course to assist individual wildlife rehabilitators and other organizations to perform faecal diagnostics. To diagnose faeces, it is essential to have access to a proper microscope (good optics is the most important factor), and photo-documentation greatly assists. However, observing various physical properties of faeces such as color, texture and odor can be a good macroscopic indicator of potential problems. The best teacher is experience, which I have gained by volunteering at Kanyana. Whilst we are still in the early stages of collecting data, I want to share our experiences so that other wildlife rehabilitators may benefit.

The Kanyana Microscopy Station was started in 1994 by Ruth Haight RVN in order to provide diagnostic support for Kanyana Hospital. Ruth's previous training in domestic animal veterinary microbiology was enhanced in 1997, when she completed an extramural program at the Perth Zoo. There, she increased her knowledge of wildlife veterinary microbiology through the aide of the zoo's vet nurses and greater resources. The first Kanyana microscope consisted of a standard lab-quality Olympus monocular scope with 4x, 10x, 40x and 100x(oil) objectives, and used a mirror as the light source. It had a mechanical stage that allowed precise movement of the slide, but it had no camera. Today, new and used microscopes of this quality can be purchased for a few hundred dollars, or obtained as a donation from local schools. These are adequate for wildlife rehabilitation needs. One can also buy substitute eyepieces that have a built-in digital camera and plug into a computer, which can transform good older scopes into state-of-the-art instruments.

In 2004, Kanyana received a LotteryWest grant that enabled it to purchase a research-quality Nikon trinocular scope (binocular with a camera port) complete with a Coolpix 4500 digital camera. Output from the AV port of the camera can be fed through a video capture card and displayed on a computer monitor for teaching purposes. The camera can capture still photos and short videos, and its zoom lens allows for even higher magnification. Last year, I joined Kanyana in order to assist Ruth in the faecal diagnoses of hospital admissions. I wanted to apply my background in microbidogy and immunology, and my interests in computers and digital photography, toward a more comprehensive approach to wildlife faecal diagnosis, complete with video and photo documentation.

The goals of the Kanyana Microscopy Station are aimed primarily at improving the diagnosis and treatment of wildlife admitted to Kanyana Hospital. Admissions range from healthy orphans and escaped pets, to sick or injured animals that require immediate assessment and treatment. Faeces diagnosis is a nonintrusive way to monitor microbial flora and fauna of an animal's gut, but it has limitations because it cannot detect infections in the other organs, lungs or blood. The light microscope cannot see the smallest of microbes (viruses), nor can it detect toxins and poisons. Detection of these agents is beyond the capability of small clinical labs such as ours, and usually requires professional diagnostic services. Faeces from new admissions may show no apparent problems, yet the animal may die subsequently of other causes. In addition, faeces is the endpoint product of a long digestive process, and mainly reflects the status of the hindgut. Nevertheless, the content of faeces is usually a good indicator of the overall health of the animal. Much information can be obtained from simple wet mount preparations without staining, although certain bacteria must be examined as stained dry mounts under an oil immersion objective, in order to make a more accurate diagnosis.

Faecal samples are collected and stored in recycled film canisters under refrigeration until they can be examined microscopically (usually on two occasions per week). When in doubt, it is always better to prepare slides from freshly obtained faeces. Sometimes, the animal is not healthy enough to provide a sample upon admission, especially if it is malnourished, so one is obtained at the first opportunity. Each sample is labeled with the animal admission number, animal species, and date of collection. In the future, we plan to use custom-designed labels that allow the hospital workers to tick off boxes and fill in blanks, to provide additional information, and allow us to develop 'faecal profiles' which we can enter into a database. The admission number is already linked to a custom database (KanyanaKare) where assessment, treatment and outcome information is stored. New admissions are kept isolated until their faecal status can be determined, so that potentially contagious diseases are not introduced. It is therefore important to assess healthy as well as sick animals, so that healthy animals can be removed from quarantine and cleared for eventual release as soon as possible. Sometimes, animals which appear healthy upon admission may have subclinical problems that are about to erupt in captivity, that can be detected beforehand.

In addition to the goals of improved wildlife diagnosis and treatment, the Kanyana Microscopy Station provides further opportunities for teaching and research. By documenting faeces physical properties along with microscopic observations, including photos and videos, and then correlating this information with clinical signs and parameters such as animal species, age (if known), diet and time of year, we hope to understand better what is 'normal' and 'abnormal'. By developing case histories as examples, we can also educate other wildlife rehabilitators, veterinarians and students, to aid in their diagnoses. The ability to document observations using digital photos and videos allows wildlife rehabilitators to exchange information by email, and to seek the advice of experts. By remaining curious of the unknown and suspicious of the obvious, as any good detective would do, opportunities exist for making discoveries. Research opportunities are further enhanced through collaborations with universities and government agencies. Last year, investigators at Murdoch University were the first to describe a new species of parasitic coccidia (*Eimeria kanyana*) in the faeces of Western Barred Bandicoots housed at Kanyana.

Most 'bad guys' that we recognise in wet mount preparations can be identified by comparison to pictures in standard microbiology and parasitology textbooks. Some microbes remain mysterious (at least to us), but are presumably harmless, as they are present in animals that appear to be healthy. The real challenges in faecal diagnosis are learning to recognise the 'good guys' and to ignore irrelevant objects. Some objects in wet mounts look remarkably like worm eggs, but do not fit the description in any textbook. Close inspection at higher power, their relative size, and their distribution amongst healthy animals from a variety of species at particular times of the year, have led us to conclude these are most likely pollen grain artifacts. Most pollen grains are unmistakable, and some can be quite spectacular. Whilst pollen in the air might account for some of the distributions we see, heavy loads of specific pollen grains are likely associated with diet. Geometric crystals in the white urates of birds and reptiles are easy to detect, but are actually unwanted as they badly cloud the wet mount preparation, making important objects difficult to see. Thus, hospital personnel collecting faecal samples should be instructed to scoop up the dark stuff, and leave the white stuff alone. Sample collection is the first step in making an accurate diagnosis, so it should be done properly. Also, wet mount preparation can be a bit tricky, and previous childhood experience playing with mud is an invaluable asset in order to uniformly attain the right consistency.

The relative size of objects observed in the wet mount is an important consideration. Because bacteria are always present and fall within a narrow size range, they can be used as an internal standard for comparison, rather than trying to determine an object's absolute dimensions. Mites and worm eggs in faeces are relatively large compared to bacteria, and are easy to see at low power (100x-200x). Protozoans such as coccidia are difficult to see even at high power (400x), and a keen eye is required to spot them. Motile protozoans can be detected by their rapid jerky movements, but are nearly impossible to identify for this reason. Yeasts are easy to see under high power, being somewhat smaller than protozoans and larger than bacteria. A contagious parasitic yeast of parrots (Avian Gastric Yeast, AGY) looks very much like a large bacterial rod, and until recently, it was mistakenly called Megabacteria. All incoming parrots at Kanyanaare quarantined until they are cleared of AGY, to prevent infection of our aviaries. Whilst bacteria come in a variety of shapes and a range of sizes, even the largest among them is still smaller than AGY. Bacteria can barely be seen under 400x even on a good scope, but the camera zoom lens can enhance their microscopic details quite dramatically.

Shape of the microbe is also an important diagnostic feature. Eggs from different worms may look similar, and need to be examined more closely to identify them. Most eggs have unique features that make textbook identification of the parent worm easy. Yeasts such as AGY are rod shaped, whilst Candida (thrush) are oval. The presence of oval yeasts does not necessarily indicate a parasitic burden unless the yeast spores are budding or germinating. One often sees non-budding yeast spores passing through the gut of fruit/nectar-eating animals, especially those on hospital diets where food may sit around for awhile. Budding yeasts form pairs or small chains, at which point they may begin to form root-like structures (hyphae) as they germinate. Because budding and germination may occur during preparation of the wet mount when dry faeces is being rehydrated, one needs to be careful about conclusions. Bacteria are shaped as straight rods, squiggly rods, or spheroid cocci. The rods can be long, short, fat or thin, relative to each other within their size range. Some spore-forming bacterial rods contain spheroid endospores that bulge outward at one end or in the middle, and upon Gram-stain look like a small coccus trapped inside the rod. Cocci are generally similar in size to each other, but are always smaller than rods, which may be as small as two cocci (coccobacilli). We differentiate between 'coccobacilli' and 'diplococci' by using the camera zoom and the fine-focus knob, to look for a boundary in the diplococcus pair. Both rods and cocci can form short chains joined end-to-end. Streptococci may form longer chains, whilst Staphlococci may form clusters, an important diagnostic distinction.

Motility is another criterion to observe, and this can best be documented using digital video-recording. Because the faecal suspension is in a liquid phase, heat from the microscope light excites the water molecules and causes small particles like bacteria to vibrate in place (Brownian motion). This should not be confused with true motility, which takes many forms. Motile microbes are quite impressive as they dart, tumble and spin, or swim like snakes. The depth of the wet mount allows motile organisms to move in and out of focus, so still photography is difficult. Motile protozoans are usually more irregularly shaped and larger than motile bacterial rods. Some motile organisms 'come alive' in the wet mount, while others (eg, obligate anaerobes) may die soon after defecation, and their motility can only be observed in fresh faeces.

In the preparation of wet mounts, enough water is mixed into the faeces to produce a suspension, and then solids are separated away in order to spread a thin liquid film under the coverslip. Most of what remains in the film are particles of food debris, urates (in birds and reptiles), and microbes. Most of the microbes are bacteria. Usually, there are so many bacteria in wet mounts that 'pattern recognition' of the microbial population becomes the aim, not

identification of individual bacteria. Quantitative assessment of bacterial load is not always useful, as the number of bacteria can vary among different wildlife species depending on their diet. It can also vary among wet mount preparations, andbe affected by the physical condition of a faecal sample. Qualitative assessment is more useful. In virtually every healthy animal we have examined, there is a diverse mixture of bacterial shapes, sizes and motility. This natural balance of microbes is the way it should normally be in all animals, similar to the ecology of any wildlife population.

Faeces from animals with gut infections almost always contain only one or two types of bacteria (monocultures). Usually, monocultures are apparent from the prevalence of mainly one type of bacteria in the liquid phase of faecal preparations. However, it is sometimes necessary to look at squashed solids in a thicker wet-mount, especially when the faeces is slimy or stringy and doesn't want to mix well with water. In these samples, we have observed slimy sheets (biofilms) of bacteria. Biofilms usually indicate a serious infection of the gut lining, and are seen in AGY infected parrots. In carrion-eating animals, biofilms may be associated with their diet (yuck!), and not be a cause for concern. This is why it is important to know the diet history of a questionable animal. Since this is not always possible with new admissions, follow-up faecal diagnosis should be performed once an animal is on a defined diet under care. Although we do not routinely perform Gram stains for diagnosis, there are occasions when this is necessary in order to provide appropriate treatment. Gram-negative and Gram-positive bacteria can be differentiated by a relatively simple staining technique, which is well described in microbiology textbooks. Otherwise, there is relatively little else that one can do to identify bacteria in small clinical labs.

III. Case History: Gut Infection in a Western Gray Kangaroo Joey

About one year ago, a vet nurse on staff at a local veterinary hospital approached Kanyana about diagnosing faecal samples from five Western Gray Kangaroo joeys under her care, who had severe diarrhoea. She had been successfully rehabilitating 5 joeys that were relatively healthy, until she took in a sixth one. The newcomer joey was over a year old, but was underweight (2.9 kg), had diarrhoea, was on Baytril antibiotic treatment, and had previously been treated with Fungalin. The diarrhoea spread to 4 of the 5 joeys (an older joey did not get sick), and she grew concerned. Three of the four original joeys were about 6 months old, on M150 Biolac milk, and not yet eating solids. The fourth sick joey was about 8 months old, on Biolac M200, and just beginning to nibble solids. The 5 samples ranged in textures from runny to pastey, and all had a fermented odor about them. Two of the 3 original joeys on M150 had light green soft stringy poo, and the other two joeys (one on M150, one on M200) had light green runny poo. The newcomer joey's poo was runny and blackish-green, a potential sign of bleeding in the upper gastrointestinal tract.

Wet mounts of the 5 faecal samples were examined and photographed. Faecal samples from the 2 joeys with soft stringy poo showed a predominance of rods, but the rods appeared diverse and the bacterial load was not that heavy. The stringy texture appeared to be due to the presence of polyester fibres in the faeces, which could not be digested. The fibres were clumped together with faecal material, and most likely were ingested when the joey groomedits pouch fabric. Most of the fibres were excluded during wet mount preparation, so those present under the slide coverslip were only a fraction of those in the faeces. Faecal samples from the 3 joeys with runny poo showed a monoculture of strangely-shaped rods. All rods were relatively long, some were flat, and some were shaped like spindles or the soles of shoes. Thus, depending on their orientation to the slide, they could appear fat (top view), thin (side view), or oval (end view). Interestingly, faeces from one of the younger joeys that had a heavy

monoculture, also contained material that looked like intestinal lining. This joey died a few days later.

Before the joey died, a faecal sample had been sent to a veterinary pathology service for analysis by wet mount microscopy, Gram staining, and cultures for aerobic and anaerobic bacteria. Comments in the lab report about the wet mountwere unremarkable. In the Gram stains, the report noted that there were a few white blood cells, very occasional red blood cells, heavy but mixed bacteria, and that no fungal elements wereseen. There was no significant growth of aerobic bacteria, and no anaerobic pathogens were isolated. The cultures were negative for *Salmonella*. I performed a post-mortem on the dead joey, assisted by the vet nurse, her partner, and a staff veterinarian. Parts of the gut were bloated like a balloon with gas, and the intestines were twisted and tangled. Spots of disease were on the intestines where the wall was thin and membranous, rather than thick and musculature as it normally should be. Gut contents were found inside the abdomen (peritonitis), but it is possible that the intestine was punctured unknowingly during post-mortem surgery. Gut contents were collected. The tip of the liver had a yellowish, discolored edge. The gut contents, sections of diseased intestine, and the liver tip were preserved in formalin for histopathology. Months later, the tissues were finally processed. The intestine shows areas of severe inflammation and ulceration, and the liver shows areas of hemorrhagic necrosis. Blood clots within the liver sinusoids also contain small dark particles which appear to be stained bacteria that were inside the bloodstream (sepsis).

After the necropsy was finished, we reexamined the Gram-stained slides using a microscope at the clinic where the post-mortem was performed. The faeces had been spread a bit too thickly, making it hard to see individual bacteria. Within the thinner clumps, there appeared to be predominantly Gram-positive cocci, dipplococci, and rods. Then, the staff veterinarian observed something that provided us with a new clue. One area of the slide displayed individual spore-forming Gram-positive rods. Looking again at the clumps, we reinterpreted what we saw previously. It then appeared that the cocci and dipplococci might be free spores, presumably released by the rods around them. Some of the dipplo-spores appeared to be encased in a shell, suggesting that these might be the walls of two spore-forming rods, joined end-to-end. All of us who saw the slides concluded that the bacteria on them were predominantly spore-forming Gram-positive rods. Because the other joeys' lives were still in danger, and we wanted to interpret the data we had obtained, I consulted clinical microbiology textbooks, online databases (PubMed), and web sites in an attempt to identify the bacteria. Based on the case history and available evidence, I concluded that the spore-forming bacterial rods are probably *Clostridium difficile*, a slow-growing, obligate-anaerobe which causes severe diarrhoea and pseudomembranous colitis.

C. difficile is closely related to some of the most toxic pathogens humans have encountered, although other *Clostridium* species are part of normal gut flora and may in fact serve a beneficial purpose. Toxic *Clostridium* species include *C. tetani*, the bacterium responsible for tetanus (lockjaw), which is caused by an exotoxin it secretes. *C. perfringens* is the cause of muscle necrosis (gas gangrene), the major infection of war casualties cured by penicillin. *C. botulinum* causes food poisoning in canned meats and beans that are not sterilised properly. The gas it produces pops up the top of the can or jar lid, an important warning sign not to eat its contents. The exotoxin it produces (botox) is one of the most potent known to mankind. *C. difficle* is actually a normal resident of human gut flora, and although it produces an inflammatory exotoxin in the colon (enterotoxin), it generally does not cause problems because it is found in low numbers. It is a poor competitor against other gut anaerobes, and usually represents only a small minority of the microbial population.

C. difficile can take over and become a serious problem when there is a microbial imbalance in the colon, which happens frequently following antibiotic therapy. Whilst even brief exposure to any single antibiotic may cause *C. difficile*-associated diarrhoea, a prolonged antibiotic course and/or the use of two or more antibiotics together increases the risk of disease. Certain antibiotics are highly correlated with onset of this disease, although it may take weeks or months after treatment before symptoms become pronounced. One of these is Baytril (enrofloxacin), a fluoroquinolone mainly used to treat respiratory infections in birds, which is routinely administered to injured wildlife in the Kanyana hospital. The USA Food and Drug Administration has banned its use in poultry because it encourages the growth of drug-resistant bacteria which are pathogenic to humans. Another antibiotic correlated with this disease is Antirobe (clindamycin), which kills many anaerobic bacteria strains, but has no effect on *C. difficile*. Thus, Antirobe mainly kills anaerobic competitors that would keep *C. difficile* in check. Antibiotics that are effective in controlling *C. difficile* are Flagyl (metronidazole) as a first choice, and if necessary, vancomycin. However, vancomycin-resistant enterococci (VRE) are a serious problem in hospitals, as they are resistant to all known antibiotics.

The enterotoxin produced by *C. difficile* causes inflammation of the intestine, resulting in the appearance of polymorphonuclear leukocytes in the faeces, which I observed. At the site of inflammation, a coagulated serum exudate covers the intestinal lining and forms cast impressions of the membrane structure (pseudomembranes), which also is strikingly similar to what I observed in the wet mount preparation. Recently, a new highly toxic strain of *C. difficile* has become one of the most feared hospital-acquired (nosocomial) infections, because it is gaining resistance to many broad-spectrum drugs, and its spores are easily spread and survive for months. The spores can withstand routine hospital disinfectants and laundry detergents, and when ingested, it can pass through human stomach acid safely. Once *C. difficile* becomes established, it causes intestinal ulceration similar to that observed in the dead joey's tissue section. The ulcers allow gut bacteria and enterotoxins to enter the bloodstream (sepsis), which is nearly always fatal.

Because severe diarrhoea due to microbial imbalance sis often made worse by further antibiotic treatment, probiotic therapies are increasingly being tried. Most probiotics (eg, Protexin) contain live freeze-dried *Lactobacillus* and related *Bifidobacterium* species, the same beneficial species found in natural yoghurt. These species are non-spore-forming, Gram-positive facultative anaerobes which produce lactic acid, rather than gas. Lactobacilli are found naturally in the female genital tracts and mammary ducts of diverse mammalian species, and are normally present in unpasteurised milk. These bacteria digest lactose, which is why natural yoghurts and cheeses are nearly lactose free. However, the name *Lactobacillus* was given because they produce lactic acid, and not all Lactobacilli digest lactose. *Clostridium* species generally cannot grow under acidic conditions. This is why steam pressure sterilisation is used for the vacuum canning of nonacidic foods such as meats and beans, whilst a boiling water bath is sufficient for acidic foods such as tomatoes and citrus fruits, or non-citrus fruits to which lemon juice has been added.

I advised the vet nurse to blend a small dab of naturally fermented ABC yoghurt containing *Lactobacillus acidophilus*, *Bifidobacterium* species, and *Lactobacillus casei*, into the Biolac milk 30 minutes before warming and feeding to the joey If possible, the *L. casei* bacteria used to make the yoghurt should contain a related species called *Lactobacillus rhamnosus GG*, which recent human studies have shown to have the greatest benefit. ABC yoghurt contains live cultures, but the bacteria have already digested their available food and have gone dormant (stationary). The 30 min incubation and warming activates the ABC bacteria in the presence of fresh food - milk. It is important that the milk formula contains lactose (eg, Biolac and

Wombaroo), because lactose-free formulas (eg, Divetalac) can provide nutrition for competing bacterial species. Because kangaroos are foregut fermenters, similar to eutherian ruminants (eg, cows), the activated bacteria should colonise the anaerobic foregut and establish conditions that discourage the growth of *C. difficile*. In hindgut fermenters and omnivores, which have acidic stomachs, the ABC bacteria might better survive passage through the stomach if they are present in the milk as it curdles, and they should have a better chance of colonising the anaerobic hindgut (eg, colon) if there is food (ie, lactose) for them to eat when they arrive. Cases of lactose intolerance in humans can often be cured by yoghurt-milk, as the lactose will be completely digested by these bacteria once inside the gut.

The vet nurse began feeding her remaining joeys yoghurt-milk for a day or two, but then stopped, because the head veterinarian of the clinic where she worked told her that yoghurt was meant for humans and not kangaroos. Actually, all marsupial milk formulas are derived from cow's milk solids, and their constituents are adjusted to coincide with the changing nutritional needs of the growing joey. There is nothing in yoghurt that is bad for the joey, and one could argue that it is the milk formulas which are lacking. Although these formulas are nutritionally complete, they are sterile and do not contain lactobacilli that would normally be present in mother's milk. Powdered milk formulas are also lacking in milk fats and colostrum which are present in the whole cow's milk used to make yoghurt. Cows are eutherian ruminants, which have a similar digestive system to foregut fermenters such as the kangaroo. Both animals depend on anaerobic bacteria to ferment the cellulose-rich plant material they eat. The metabolites released by these bacteria provide nutrition for other microbes. The foregut bacteria become food for larger protozoans that synthesize high-quality protein, and the protozoans are easily digested in the animal's lower intestine.

The remaining joeys recovered; however, it is unclear whether yoghurt-milk made a difference. Whilst yoghurt-milk may suppress *C. difficile* infections, this is only one cause of diarrhoea which can successfully be treated with ABC yoghurt. Since the death of that animal, I have been asked by other rehabilitators to diagnose joeys that have diarrhoea. After ruling out identifiable pathogens as the cause, I advised them to try yoghurt-milk therapy. The first person who took my advice was Sue Turner, a highly experienced marsupial rehabilitator who volunteers at Kanyana. Remembering the contrary advice given by the veterinarian, I wasn't sure whether yoghurt-milk therapy would work, and asked her to provide me with feedback. A few days later, I was very pleased to get a note from her, saying "Hi Gerry, just to let you know I started my joey Millie on ABC yogurt as suggested for diarrhoea. In just 24 hrs, there was a considerable improvement. By the third day, the diarrhoea had gone. All are looking good. Millie is 1.8 kg, so I added 1 ml of yogurt to her first and last bottle. Regards, Sue - PS. Thanks from Millie also!!" Since then, other joey rehabilitators have followed my advice and reported back positive results. Yet, when I discuss the issue of probiotics with medical and veterinary personnel, many remain skeptical, as if this were some New Age hocus pocus. Actually, yoghurt therapy for the treatment of gut and vaginal infections was advanced by the Nobel Laureate Elie Metchnikoff around 100 years ago, but it stopped being commonly used after the discovery of penicillin.

It is clear that antibiotics, when used properly, can make the difference between life and death. However, it is also becoming evident that their immediate benefit may be a tradeoff, because of their long-term side effects on the body's microbial ecology. It has been estimated that there are over 1000 different bacterial species living on the human body. All bodily surfaces exposed to the outside world – skin, eyes, ears, mouth, airways, gut, anus, urogenital tracts, mammary ducts – are covered with microbes. Therefore, following antibiotic therapy to get rid of the 'bad guys', it is important that we reintroduce the 'good guys'. Some 'good guys' have already been

studied, but we are far from understanding microbial ecology well enough to restore an altered balance to its natural healthy state. Sadly, through this joey's unfortunate death, I have learned important lessons that may not only help save the lives of my wildlife friends, but my human friends as well.

I am almost certain that the sick joey newcomer transmitted its disease to the previously healthy ones. This could have occurred through a number of routes. Because *C. difficile* spores could contaminate fabric pouchs, the way these are laundered could make a difference. One could reduce the spore load by methods such as disinfecting first in bleach, washing in very hot water, and being compulsive about washing separately each animal's pouch, etc. However, the animals will groom each other anyways, and unless sick animals are quarantined, microbial transmission is unavoidable. Even after disinfection, the pouch material itself may contribute to anaerobic diseases by creating an unnatural substrate in the intestine, especially if polyester pouches are not lined with natural fabrics such as cotton and silk. However, at least in the case of *C. difficile*, a major known risk factor is the use of antibiotics.

Conclusion

In a wildlife hospital setting, such as Kanyana, the use of anti-bacterial, anti-fungal, anti-protozoan, and anti-worm medications is an unquestionable 'necessary evil'. Contagious microbes are forbidden in our facility for obvious reasons, and sick or injured animals must be treated. For the sake of hygiene and in order to maintain a pathogen-free hospital environment, any animal whose faeces contains evidence of parasites or infections is treated appropriately. However, this case history has raised a number of questions in my mind about our approach to wildlife medicine, particularly with regard to the rehabilitation of mammals. Unless the animal is injured, unhealthy or has a heavy parasitic burden, should we treat prophylactically? Should probiotics be used more frequently? What is the effect of treating with broad-spectrum antibiotics on an animal's microbial ecology? Are we inadvertently selecting for drug-resistant microbes, similar to what happens in human hospitals? Could some of these microbes be zoonotic, or transfer their drug resistance to human bacterial pathogens? What is the effect on the microbial ecology of wildlife populations because we release treated animals, and should there be uniform antibiotic treatment standards for wildlife rehabilitation? In time, I am sure that some of these questions will be answered by us and others.

BIOGRAPHY

Gerry Waneck PhD came from Boston USA to Perth in Nov 2005, to be Head of the Transplant Immunobiology Laboratory at Sir Charles Gairdner Hospital, and is an Associate Professor in the School of Medicine and Pharmacology at UWA. Gerry sought to apply his strong biomedical background and history of environmental activism to assisting Australian wildlife conservation organizations. He joined Fauna Rehabilitation Foundation in January 2006 as a volunteer, and was attracted to Kanyana Wildlife Rehabilitation Centre in June 2006. At Kanyana, Gerry assists in faecal diagnostic microscopy, education and outreach, and is a member of the computer IT team.