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Mycotoxins in food products

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Mycotoxins are toxic metabolites produced by fungi, especially by saprophytic moulds growing on foodstuffs or animal feeds. They must always have represented a hazard to man and domestic animals, but their effects have been largely overlooked until the past 20 years. Although poisonous mushrooms are carefully avoided, moulds have generally been considered to cause unsightly spoilage of food, without being dangerous to health. However, it is now well established that mycotoxicoses (diseases caused by mycotoxins) have been responsible for several major epidemics in man and animals, at least during recent history. The most important have been: **ergotism**, which has killed hundreds of thousands of people in Europe in the last millenium; **alimentary toxic aleukia** (ATA) which brought slow death to tens of thousands of people in the USSR between 1941 and 1947; **stachybotryotoxicosis**, which killed tens of thousands of horses and cattle in the USSR in the 1930s; and **aflatoxicosis**, which killed 100,000 young turkeys in England in 1960 and has caused death or disease in many other animals – and perhaps man as well.

Each of these diseases is now known to have been caused by growth of specific moulds which produced one or more potent toxins in a particular commodity.

An important distinction must be made between bacterial toxins and mycotoxins. The classic bacterial toxin is a protein which swiftly produces antibody reactions with characteristic symptoms. Fungal toxins are almost all low molecular weight chemical compounds and hence are unsuspected and insidious in their action. Mycotoxins can be acutely or chronically toxic, or both, depending on the dose and the kind of toxin. In animals, symptoms of acute toxicity include liver and kidney damage, attack on the CNS, skin disorders, and hormone-like effects. Nerve toxins may induce trembling or death without apparent cause. Skin disorders may be manifest as open lesions or as photosensitivity, while the manifestation of hormonal effects include abortion in cattle, vulvovaginitis in pigs, and a variety of ill-defined disorders such as vomiting, feed refusal and ill thrift. Toxins producing liver and kidney damage are even more insidious: levels much lower than those producing acute effects are often carcinogenic. Ingested in small quantities in the diet, they can cause cancer in experimental animals long after the time of ingestion. It is probable that man can be affected in the same way.

Significant mycotoxins

Table 1 lists a number of moulds, some of the mycotoxins they produce, and known or possible acute diseases in which they have been or are involved. There are at least 10 human diseases which are now known to be, or are suspected of being, caused by mycotoxins. Each of them is briefly reviewed.

Ergotism

The association of one human illness with a fungus has been known for a long time. Ergotism occurred throughout the last millenium in central Europe, and has certainly killed hundreds of thousands of people. The relationship between ergotism and the formation of ergots in maturing grain was established by the 17th century. By 1750, it was known that ergots result from the growth of the fungus *Claviceps purpurea* in the ovaries of grains, especially rye. During milling, ergots are not readily separated from sound grain and as a result become fragmented and dispersed throughout the flour.

The first symptom of ergotism is a feeling of coldness in the hands and feet, followed by a sensation of intense burning. In extreme cases, gangrene, necrosis and death may follow. In the Middle Ages the disease was known as 'St Anthony's Fire', because it was believed that travelling to the shrine of St Anthony would relieve the burning sensation. Modern medicine is more likely to attribute the curative effect of this trip to the victim moving away from his contaminated environment.

The toxic principles in ergots are now known to be a range of alkaloids, all derivatives of lysergic acid, which have a wide spectrum of biological activities. Some have been used in low doses for many years to induce childbirth.

The last known outbreak of ergotism occurred in the French village of Pont St Esprit in 1954. More than 200 people became ill and four died from cardiovascular collapse as a result of muscular spasms. This well-documented mycotoxicosis[1] was due to gross negligence by a miller. It was notable because many people suffered from hallucinations, screaming that they were on fire or were being chased by wild beasts. Fuller suggests that the alkaloid responsible for these symptoms may have been lysergic acid diethylamide (LSD).

Ergotism can now be regarded as a disease of the past since stringent controls on levels of ergot in grain have been established throughout the world.

Acute cardiac beriberi

Another human mycotoxicosis of significance is acute cardiac beriberi. It was a common disease in Japan, especially in the second half of the 19th century.

The first symptoms of acute cardiac beriberi are heart distress and palpitation, with rapid breathing. After a few hours, breathing becomes laboured, nausea and vomiting are experienced, and within two to three days, anguish, pain, restlessness and sometimes maniacal behaviour occur. In extreme cases, progressive paralysis leading to respiratory failure may cause death.

Beriberi is the general name given to a vitamin B_1 deficiency resulting from the consumption of polished rice. However, painstaking and pioneering work by Uraguchi[2] established that acute cardiac beriberi probably was not an avitaminosis but a mycotoxicosis. In 1910, the incidence of acute cardiac beriberi suddenly decreased: Uraguchi points out that this coincided with implementation of a government inspection scheme which dramatically reduced the sale of mouldy rice in Japan. The incidence of true beriberi was unaffected. It is notable that the victims of acute beriberi were often young adults without any history of disease.

Alimentary toxic aleukia

A third disease caused by a mycotoxin is known as alimentary toxic aleukia (**ATA**). From 1942 to 1948 this disease caused the deaths of many people in Russia, especially in the Orenburg district north of the Caspian Sea.[3] In some localities, mortality was as high as 60% of those afflicted and up to 10% of the population. The Russian authorities have apparently never released a figure for the total mortality, but it must have been at least one hundred thousand. Records show that ATA was prevalent in 1932 and 1913, and, no doubt, outbreaks occurred in earlier years as well.

ATA is an exceptionally nasty disease: symptoms include fever; haemorrhagic rash; bleeding from the nose, throat and gums; necrotic angina; extreme leucopenia; agranulocytosis; sepsis; and exhaustion of the bone marrow. These symptoms are surprisingly similar to those caused by radiation poisoning and contrast with those caused by bacterial toxins or other mycotoxins.[3]

Outbreaks of ATA were always associated with bread and other cereal products made from grain that was allowed to remain unharvested over the winter months. During and after World War II, agricultural labour in Russia was very limited and caused delays in harvesting. Food was often very scarce, which resulted in the consumption of poor quality grain.

Russian studies in the 1950s suggested that fungi may have been involved in ATA and that

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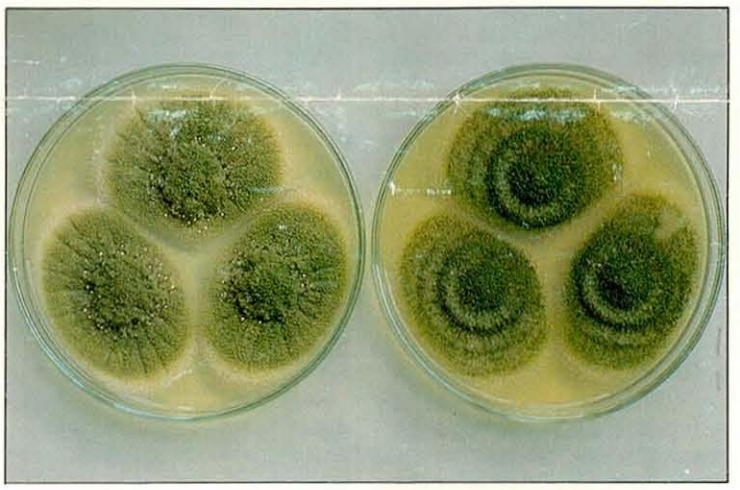

Figure 1: Colonies of Aspergillus flavus on Czapek yeast extract agar and malt extract agar after growth for 7 days at 25°C.

Table 1. Acute mycotoxicoses of human significance.

Date	Disease	Toxin	Cause	Diagnosis
to 1954	ergotism	ergot alkaloids	*Claviceps purpurea* in rye	1800: fungal cause suspected 1850: fungal cause demonstrated
to 1910	acute cardiac beriberi	citreo-viridin	*Penicillium citreo-nigrum* in rice	1910: yellow rice sale banned 1969: fungal cause demonstrated
to 1948	alimentary toxic aleukia	T-2	*Fusarium poae* in millet and rye	1950: fungal origin suspected 1976: toxin established correctly
1965–66	'cobalt-beer' cardiomyopathy	T-2	*Fusarium spp.* in barley	1980: fungal origin proposed
1974	hepatitis	aflatoxin	*Aspergillus flavus* in maize	1975: fungal cause demonstrated
current	pellagra	T-2	*Fusarium spp.* in maize	1980: fungal origin proposed
current	Reye's syndrome	aflatoxin	*Aspergillus flavus* in nuts and maize	1977: fungal origin proposed
current	kwashiorkor	aflatoxin	*Aspergillus flavus* in nuts and cereals	1983: fungal involvement proposed

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alternate freezing and thawing of the grain was essential for toxin production. However, early studies on the causes of ATA were inconclusive. During the 1970s it became clear that ATA was a mycotoxicosis caused by the trichothecene toxin known as T-2. Trichothecenes are a class of toxins produced by *Fusarium* species when they grow in grains, and by some other related fungi.

Trichothecenes have emerged in the past 10 years as by far the most significant mycotoxins. Apart from ATA, they are now believed to have been involved in a variety of diseases of man and domestic animals. Most of these have occurred in Europe, the USSR, Japan and the United States.[4] Toxicity is usually acute, but it has now been suggested [5] that trichothecenes may be implicated in the high incidence of oesophageal cancer that occurs in the Republic of Transkei (South Africa).

Table 2. *Chronic mycotoxicoses of human significance.*

Date	Disease	Toxin	Cause	Diagnosis
current	liver cancer	aflatoxin	*Aspergillus flavus* in foods	1963: fungal cause unknown 1970-75: epidemiological studies 1977: statements of human risk
current	oesophageal cancer in Transkei	deoxynivalenol, zearalenone	*Fusarium spp.* in maize	1979: fungal cause suspected
current	nephropathy in Denmark	ochratoxin	*Penicillium viridicatum* in barley fed to pigs	1973: fungal cause known in pigs: suspected in humans from residues in pork
current	Balkan endemic nephropathy	?	?	1972: fungal cause suspected

Other possible acute mycotoxins

Table 1 also lists some human diseases in which mycotoxins may be involved. Pellagra is a skin disorder, accompanied by severe mental disturbance and is almost entirely confined to people who subsist on maize of poor quality. Frequently, such maize is visibly mouldy. Pellagra has been considered for more than 50 years to result from vitamin B deficiency, but Schoental [6] presents persuasive evidence that it is due to the growth of *Fusarium* species on moist corn with the consequent formation of T-2 and other trichothecene toxins.

Schoental also mentions outbreaks of disease among heavy beer drinkers in Quebec, Minnesota, Nebraska and Belgium in 1965–66. In Quebec, this syndrome became known as cobalt-beer cardiomyopathy. A number of deaths occurred. However, the accepted explanation of cobalt poisoning (arising from its use as an antifoam agent) is untenable in Schoental's view because of the low levels of that metal (0.5mg/l) in beer. Indeed he points out that the symptoms of this disorder were not dissimilar to those of pellagra.

Each of the diseases discussed above and listed in **Table 1** is an acute disease syndrome. With the exception of the possible involvement of trichothecene toxins in cancer of the oesophagus, the mycotoxins involved are not known to have teratogenic, mutagenic, carcinogenic or other long-term effects.

The following set of mycotoxicoses (**Table 2**) may involve acute effects, but their more significant disease implications relate to their chronic toxicity. The best known and most thoroughly studied of these toxicoses are caused by aflatoxins.

Aflatoxicosis

Aflatoxins were discovered in 1960 following the deaths of 100,000 turkey poults in England and a high incidence of liver disease in ducklings in Kenya and hatchery-reared trout in California. Perceptive workers in England (see Stoloff [7] for a review) established that these problems were caused by toxins produced by the common moulds *Aspergillus flavus* (**Figure 1**) and *A. parasitucus*. They had devised an assay and had carried out preliminary toxicological studies by 1963. The most potent aflatoxin, B_1, is usually associated with aflatoxin G_1 and smaller amounts of aflatoxins B_2 and G_2 in natural products. Aflatoxin M_1 is produced during mammalian metabolism of B_1, and is secreted in milk. Aflatoxins have both acute and chronic toxicity to animals, and they produce the following four distinct effects:

— acute liver damage;
— liver cirrhosis;
— induction of tumours; and
— teratogenic and other genetic effects.

Fortunately, acute toxicity of aflatoxins to humans has been rarely encountered.[8] In 1967, 26 Taiwanese from two farming communities became ill with apparent food poisoning. Nineteen of these were children, of whom three died. Rice from affected households was blackish green and mouldy, and appeared to be of poorer quality than that from households that were unaffected. Samples of the mouldy rice contained about 200μg/kg of aflatoxin B_1 which was probably responsible for the outbreak: post-mortem examinations were not carried out. In 1974, an outbreak of hepatitis that affected 400 Indian people, of whom more than 100 died, almost certainly resulted from aflatoxins.[9] The outbreak was traced to corn heavily contaminated with *Aspergillus flavus*; it contained up to 15mg/kg aflatoxin. Consumption of aflatoxins by some of the affected adults was estimated to be 2–6mg in one day: it can be concluded that the acute lethal dose for adult humans is in the range of 1–10mg. It is suspected that Reye's syndrome, a common cause of death in South-East Asian children, and kwashiorkor, usually attributed to nutritional deficiencies in Northern Africa, may be related to aflatoxin intake. Evidence is not conclusive, however.

Aflatoxins and primary liver cancer

The first warnings that aflatoxins might be able to cause human liver cancer came scarcely two years after their discovery. This disease has a high incidence in central Africa and south-east Asia. When epidemiological evidence suggested a possible correlation with mycotoxins in the food supply, field studies were initiated on an international basis. Epidemiological data were coupled with analyses of those foods that form the staple diets of stable indigenous populations. Stability in both diet and population is essential in studies of this kind because of the long induction period (10–20 years) for human liver cancer.

Such studies were carried out in Kenya, Swaziland, Uganda, Thailand, Mozambique and rural south-east United States. Great care is needed to obtain meaningful results in such studies, but by 1976 sufficient data existed to allow plotting and statistical analysis.[10] These indicated a positive correlation between the logarithm of aflatoxin intake and the occurrence of human primary liver cancer, at least in Africa and south-east Asia.

Epidemiological studies in the USA have produced results differing from those above. Stoloff and Friedman [11] estimated that children in rural communities in the southern states of the USA may ingest as much as 40ng aflatoxin per kilogram of body weight per day, mostly from maize. From van Rensburg's figures, such a level should produce 4–10 deaths from primary liver cancer per 10^5 population per year. The actual level encountered, however, is about one. That is less than in some other regions of the USA, such as Wisconsin and California, where aflatoxin is unlikely to be ingested in significant amounts.

Ochratoxins

In the early 1970s, observers in Denmark noted a high incidence of nephritis (kidney inflammation) in pigs at slaughter. A search for possible causes eventually showed the presence of ochratoxin A, a mycotoxin originally reported from the common mould *Aspergillus ochraceus*. Analysis of pig feeds showed that 50% of samples contained ochratoxin A at levels up to 27mg/kg. The mould responsible was found to be *Penicillium viridicatum*, which often occurs in Danish barley.[12]

Danish law ensured that nephritic kidneys were condemned, but this did not result in the rest of the carcass being declared unfit for human consumption. The discovery of ochratoxin led to analyses of pork and bacon. These showed that a significant proportion of ingested ochratoxin lodged unchanged in body fat. The risk to humans is difficult to assess, but since pig meats are popular in Denmark and rural populations would usually eat their own, uninspected pigs, a risk has certainly existed for some time. Death rates owing to kidney failure are high in some Danish rural areas and it is a reasonable hypothesis that the cause is ochratoxin.

Balkan endemic nephropathy

With evidence that ochratoxin may be involved in deaths in Denmark, European scientists turned their attention to a disorder of unknown and baffling aetiology, known as Balkan endemic nephropathy, which occurs in eastern Europe. This disease has a long history in certain parts of Yugoslavia and Romania. Whole families have been struck down, resulting in towns with a proportion of houses boarded up because people could not be induced to occupy them after the mysterious deaths of the original inhabitants. At least one small Yugoslav town has been moved to a new location to avoid the population being wiped out.

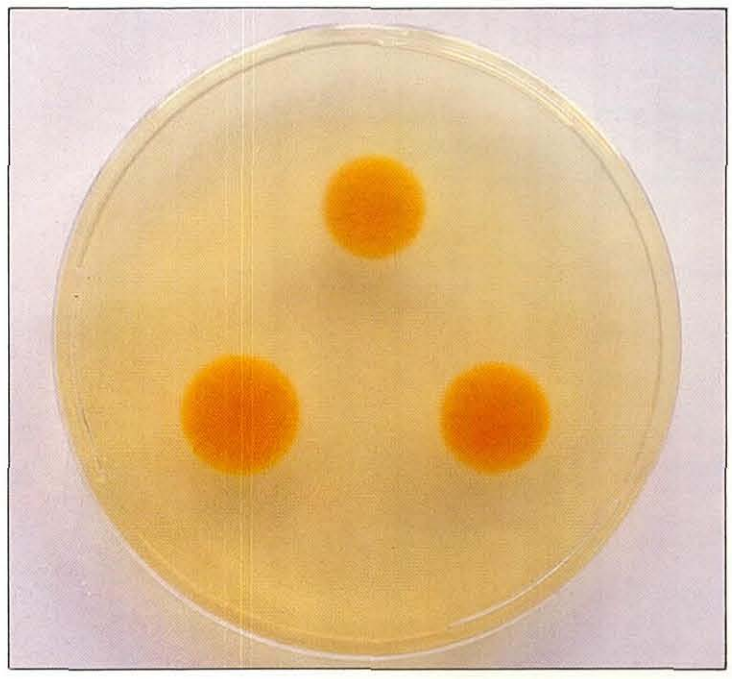

Figure 2: Orange-yellow reverse of colonies of Aspergillus flavus when grown on Aspergillus flavus and parasiticus agar for 2 days at 30°C.

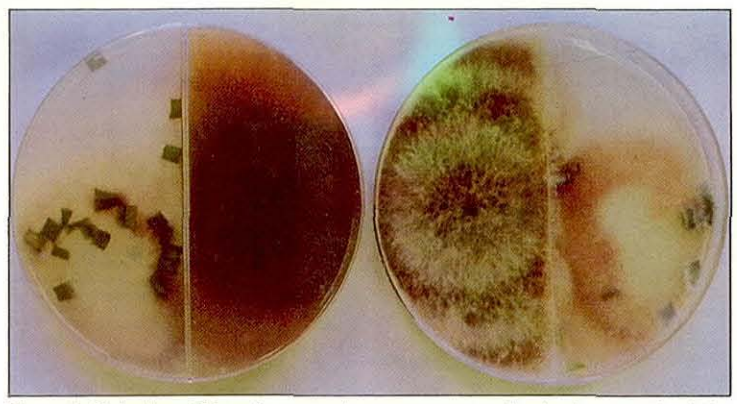

Figure 3: Colonies of Fusarium graminearum on carnation leaf agar and potato dextrose agar. Note the deep red reverse on PDA.

The disease remains baffling, but the absence of any known causative agent and the presence of the nephropathic syndrome suggest that a mycotoxin may be to blame.[13] Studies are continuing.

Assaying mycotoxins

Mycotoxins can be assayed by either chemical or biological techniques. The most common chemical technique is thin layer chromatography of solvent extracts, followed by visualisation by ultraviolet light or chemical reagents. Quantitative techniques have been developed for most common mycotoxins. However, trichothecenes have proved to be especially difficult to assay by this technique. The best procedures rely on such sophisticated techniques as gas-liquid chromatography coupled with mass spectrometry. Although of great value for the assay of specific compounds, chemical methods cannot provide a guarantee of non-toxicity because the existence of unknown toxic compounds cannot be taken into account.

Biological test methods include the feeding of suspect materials or extracts to test animals and the injection of extracts into the yolk sacs of fertile eggs. Biological tests have the advantage that definite answers on the toxicity of a food or feed can be provided. However, they are non-specific, rarely providing information on toxin type, and are slow and very expensive.

Overall, mycotoxin assays remain difficult, requiring a high level of operator skill and expertise, and are time-consuming and relatively expensive.

Detecting mycotoxigenic fungi

The detection of mycotoxigenic fungi is also difficult because so many kinds of fungi make mycotoxins and the identification of fungi remains a specialist task. Ideally, a selective medium should be available for each toxigenic fungus in the same way that selective media have been formulated for specific food poisoning bacteria. Development of such media is in its infancy: at this time only two media of this type exist, one for *Aspergillus flavus* and *A. parasiticus*, and one for *Penicillium viridicatum* and *P. verrucosum*, which make ochratoxin and some other less well known toxins.

The medium for *A. flavus*, known as *A. flavus* and *parasiticus* agar (**AFPA**) [14] was developed from the observation that these fungi produce a brilliant orange-yellow reverse colour in the presence of ferric citrate.[15] AFPA contains inhibitors which control the growth of spreading fungi and an antibiotic to eliminate bacterial contamination. Petri dishes prepared by normal plating techniques are incubated at 30°C for

42–48 hours and then examined for the characteristic reverse colour (**Figure 2**). Colour formation is very specific: only *A. niger* grows as rapidly as *A. flavus* on this medium, but it does not produce the orange-yellow colour. If doubt arises, or for cases where confirmation is essential, *A. flavus* and *A. parasiticus* are readily recognised fungi in pure culture.[16] AFPA is well suited to routine laboratory use because of the relative rapidity with which results are obtained and its simplicity.

The medium for *P. viridicatum* and *P. verrucosum* is 'pentachloronitrobenzene rose bengal yeast extract sucrose agar' (**PRYS**).[17] PRYS relies on the production of a violet-brown reverse colour by these two species. The medium appears to be quite specific for these two fungi, but the colour changes are not as easy to read as AFPA.

Fusarium species, the principal producers of trichothecenes, are most readily recognised by their large crescent shaped spores and by pink to red reverse colours on potato dextrose agar (**Figure 3**). The spores are not always formed on laboratory media, however, and some trichothecene producers do not make these reverse colours. A great deal of further work is needed in the formulation of media for mycotoxigenic fungi.

Conclusion

Improved living standards, higher quality food supplies and a more varied diet have helped to virtually eliminate the risk of acute mycotoxicosis in man in most Western countries. However, there is ample evidence that mycotoxins can, and still do, cause human disease in some developing countries, particularly in rural populations whose diet is based on a single, staple commodity such as corn, rice or peanuts.

Human disease only represents the tip of the iceberg when considering the effects caused by mycotoxins. The world-wide cost of debilitating diseases and death in domestic animals due to the ingestion of mould-contaminated feed must be enormous. Such problems can never be completely overcome, but with a better understanding of the physiological effects of mycotoxins, and the fungi which produce them, they can, perhaps, be minimised.

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Listeriosis – epidemiology and bacteriology

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Listeria monocytogenes, first described in 1926 by Murray, Webb and Swann,[1] still remains a baffling micro-organism for the epidemiologist. Animals, fish, birds and humans are all susceptible to infection [2] but, despite the organism's widespread distribution in nature, human infections are comparatively rare. Furthermore, the mode and source of infection are often obscure.

In man, listeriosis produces a number of clinical manifestations — characterised initially by influenza-like symptoms – of which neonatal and adult meningitis are the most common. In addition, abortion, miliary granulomatosis, biliary atresia, internal and external abscesses,[3] subacute bacterial endocarditis and opportunistic infection in the immunosuppressed patient, have been reported.

Transmission

It is not known whether or not there is a single reservoir of infection. The organism has been isolated from dust, water, soil, fodder and silage, and many species of birds, fish, animals and insects, but little is known about its mode of transmission. In the foetus infection could occur *in utero* or during childbirth; in adults it could occur by handling contaminated material, i.e. milk and milk products or meats.

Oral or venereal infection and insect vectors have also been suggested as routes of possible transmission:[3] the isolation rate of *Listeria monocytogenes* from human stools, for example, can be as high as 29%.

Epidemiology

The epidemiology of *Listeria* infections is poorly understood and there is a shortage of information relating to the total number of bacteriologically proven cases which occur world wide.

Seeliger *et al* [4] found that the peak of the animal infections occurred in the spring, whereas the peak of the human infections was reached in the autumn months. A similar pattern was reported by Busch in the USA.[5] It is of interest that the peaks of infection in humans and animals coincide with the seasonal peak in the birth rates.

As mentioned above, there is no clear evidence as to the mode of transmission of the disease. However, there is a possible relationship between the number of infections occurring in those individuals who are in contact with animals and living in rural areas compared with those living in urban areas.

Table 1. Biochemical tests used to differentiate between the four species of Listeria.

Test	*L. monocytogenes*	*L.grayi*	*L. dentrificans*	*L. murrayi*
Voges Proskauer	+	+	–	+
nitrate reduction	–	–	+	+
acid from mannitol	–	+	–	+
acid from arabinose	–	–	+	–
acid from xylose	–	–	+	–
metachromatic granules	–	–	+	–

An outbreak of listeriosis involving 49 patients in Massachusetts was reported by Fleming *et al*.[6] Seven neonates and 42 immunosuppressed adults were infected and 14 deaths (29%) occurred. The infection was strongly associated with a single brand of pasteurised milk. Predominantly serotype 4b was isolated from the patients and the same serotype was isolated from a bulk storage tank at a milk supply farm. Four of the supply farms experienced listeriosis in their dairy cows during the outbreak. More recently, eighty-six cases of *Listeria monocytogenes* infections, including 29 deaths, were identified between January 1 and June 14 1985 in Los Angeles and the Orange Counties, California. The infections are linked with eating Mexican-style fresh cheeses from one particular manufacturer. Type 5b was isolated from the implicated cheeses. It has been suggested that pasteurisation had been inadequate or that small quantities of raw milk were present in the product.[7] There is evidence[8] that *Listeria monocytogenes* can survive the pasteurisation procedure if the organism is present in numbers exceeding $5 \times 10^4/cm^3$. Such events may be important in the assessment of transmission of sporadic listeriosis. The feeding of poor quality silage to animals has been recognised as a source of Listeria infection in animals.

L. monocytogenes has been isolated from sea birds and starlings, and it is suggested that the latter could be incriminated with causing infections in sheep by their association with sheep flocks and the consequent faecal contamination of pastures.

Antimicrobial therapy

Wiggins, Albritton and Feeley [9] tested 125 clinical isolates of *L. monocytogenes* collected from USA sources for antibiotic susceptibility. They found a homogeneous population of organisms susceptible to ampicillin, penicillin, erythromycin and tetracycline. The combination of penicillin or ampicillin with an aminoglycoside was found to be advantageous, giving earlier and more complete killing than either agent alone. This would be an advantage for immunocompromised patients and neonates.

Listeria species

Three species of Listeria have been allocated to the genus over the past years (**Table 1**) and two other strains have been suggested for inclusion: *Listeria ivanovu* is the name suggested for the haemolytic strains of serovar 5,[10] and *Listeria innocua* for the apathogenic strain.

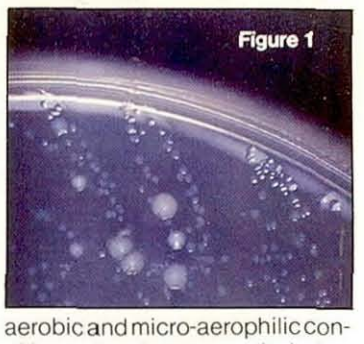
Listeria monocytogenes is a small Gram-positive bacillus, at times appearing as a diphtheroid-like rod, measuring 1.0μm to 4.0μm in length and 0.5μm in breadth. In cerebrospinal fluid smears, Listeria organisms are found intracellularly and extracellularly. They may be confused with corynebacteria, pneumococci, streptococci and, if over decolorised, *Haemophilus influenzae*.

Motility

Listeria monocytogenes is actively motile at temperatures between 18°C and 22°C. Conversely, this activity is weak or absent at 37°C. The motility is seen as characteristic tumbling and rotating motions and, if carefully employed, this phenomenon can lead to a definite identification of *Listeria* species. One other bacterial species, the Gram-negative *Pasturella pseudotuberculosis*, is also motile at 22°C and weakly motile at 37°C. However, its motion does not involve tumbling. Seeliger [11] studied a considerable number of supposedly non-motile strains and found them to be motile. It is essential, therefore, that the motility of *L. monocytogenes* is demonstrated by a reliable method.

Cultural characteristics

Listeria monocytogenes grows on most bacterial culture media used for routine culture purposes. Growth is enhanced in the presence of glucose (0.1% w/v), serum (1% w/v) or blood (5% v/v). The optimum pH range for growth is 7.0–7.4 and, although pH tolerant up to 9.0, it will not survive at pH 5.6 or below.[12] It will grow under



aerobic and micro-aerophilic conditions, the best growth being achieved in an atmosphere of slightly reduced oxygen tension and carbon dioxide enrichment.

In the absence of blood, colonies are very small after 24 hours incubation at 35°C. At 48 hours, freshly isolated strains are 1–2mm in diameter and appear as greyish-white, glistening, smooth, dome-shaped colonies. In contrast, on blood agar, colonies are 1–2mm in diameter after only 24 hours incubation and are surrounded by a narrow zone of beta-haemolysis. Non-haemolytic strains do occur, however.

When *Listeria* colonies grown on clear (non-blood) agar are examined microscopically with a beam of obliquely transmitted white light at an angle of 45° (**Figure 1**), they have a pale – blue appearance with a finely textured surface.[12] The colour and texture is so characteristic that *Listeria* colonies can be identified on heavily contaminated plates. However, some other bacterial species also produce coloured colonies in this way; for example, diphtheroid bacilli and lactobacilli produce a deep blue coloration. The principle of the

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method depends on the fact that the organisms are arranged in layers in the colony and collectively act as a diffraction grating. The size of the colony is such that under the lighting conditions used it reflects the blue part of the spectrum back to the observer.

The temperature range for growth of *Listeria monocytogenes* is 37–45°C with an optimum temperature at 30°C.[12] At 4°C, *Listeria* is believed to multiply more rapidly than other species.[13] This property has been used by Gray and by other workers to recover *Listeria* species from heavily contaminated materials and cultures. *Listeria* growth at this temperature is apparent to the naked eye after about 10 days; the organisms are motile and are pathogenic to laboratory animals.[13]

Biochemical identification

Carbohydrate fermentation patterns of *L. monocytogenes*, performed in meat extract broth with bromocresol purple indicator, incubated at 35°C for 24–72 hours are usually characteristic. Acid reactions occur with dextrose, maltose, d-laevulose, solicin, aesculin, dextrin and trehalose. No acidification occurs with mannitol, arabinose, dulcitol, adonitol, raffinose or inulin.

Seeliger [11] reported that *L. monocytogenes* does not produce indole, nor does it reduce nitrates or hydrolise urea. The production of acetymethyl carbinol is usually positive, and the methyl red test and catalase are both positive. Proteolytic activity is absent.

Using selective agents to isolate L. monocytogenes

To aid recovery of *L. monocytogenes* from heavily contaminated environments many workers have suggested the use of various agents to suppress growth of other bacterial species – especially Gram-negative organisms – and to allow the bulk of the Gram-positive species to flourish. One of the first practical observations made in this direction was the use of temperature as a selective agent. Gray *et al* [14] reported that *L. monocytogenes* not only survived at 4°C for long periods but actually grew at this temperature more rapidly than other bacteria. Chemicals used as selective agents for the isolation of *L. monocytogenes* were first recommended by Schoer [15] who suggested the use of potassium tellurite as an inhibitor of Gram-negative organisms. This finding was confirmed by Gray *et al* [16] in spite of a report that potassium tellurite could exert an inhibiting effect on *L. monocytogenes* depending upon the constituents of the broth medium.[16] Substitutes for potassium tellurite were quanofurazone,[17] lithium chloride,[18] and potassium thiocyanate.[19] Dijkstra,[20] working on the isolation of *L. monocytogenes* from silage and animal faeces, reported that nitrofurazone and 2-phenylethanol were the selective agents of choice, whilst Beerens and Tahon-Castel [21] used the antimicrobial chemotherapeutic agent nalidixic acid as a selective agent for the isolation of certain Gram-positive species. After a thorough investigation of media selective for *L. monocytogenes*, a combination of thallous acetate and nalidixic acid was recommended.[22] Leighton [23] confirmed this finding.

The use of dyes has received little attention, perhaps because of the wide variation which occurs in the manufactured products. Pancheco and Santos [24] tested nine strains of *L. monocytogenes* against 31 different dyes, the most effective of these as selective agents being nigrosin, methyl violet, crystal violet, methyl green, Sudan III, basic fuchsin and water soluble cosin. Interest in the use of dyes has been revived with reports of trypaflavine acting as a selective agent,[25] and of a medium containing polymyxin, nalidixic acid and methylene blue.[26]

The construction of media selective for *L. monocytogenes* is not straightforward as many of the selective agents suggested can inhibit the growth of *Listeria*. Furthermore, it is also apparent from the literature that a selective medium which is efficient in the hands of one group of workers will prove to be totally inadequate in other hands.

Table 2. The current somatic and flagellar antigen distribution for Listeria spp.

Designation Paterson	Seeliger-Donker-Voet	O-Antigens	H-Antigens
1	1/2a	I II (III)	AB
	1/2b	I II (III)	ABC
2	1/2c	I II (III)	BD
3	3 a	II (III) IV	AB
	3 b	II (III) IV	ABC
	3 c	II (III) IV	BD
4	4 a	(III) (V) VII IX	ABC
	4 ab	(III) V VI VII IX	ABC
	4 b	(III) V VI	ABC
	4 c	(III) V VII	ABC
	4 d	(III) (V) VI VIII	ABC
	4 e	(III) V VI (VIII) (IX)	ABC
	4 f	(III) V XV	ABC
	4 g	(III) V VI VII X XI	ABC
	5	(III) (V) VI VIII X	ABC
	6	(III) VII VIII XI	ABC
	7	(III) XII XIII	ABC
	L.grayi	(III) (XII) XIV	E..
	L.murrayi	(III) (XII) XIV	E..

Tests for pathogenicity

Pathogenic strains of L. monocytogenes can be identified by the Anton eye test. Two to three drops of an overnight culture suspended in 5ml of distilled water are introduced into the conjunctival sac of a rabbit or guinea-pig. A purulent conjunctivitis develops in 2–5 days with the organisms being demonstrated in direct films of pus. Ampicillin drops in the eye rapidly remove the infection.

Pathogenic strains may also be recognised by the *in vitro* tests described by Groves and Welshimer.[27] A positive CAMP[28] reaction, acid reaction with rhamnose and non-acid reaction with xylose were associated with pathogenic *L. monocytogenes*.

Antigenic structure

The current somatic and flagellar antigen distribution is shown in **Table 2**.[29] Serotypes 1/2a and 4b are associated with human and animal infections. Serotype 5 (*Listeria ivanovu*) has been reported as the infective agent in ovine infections.

Conclusions

It appears from literature surveys that microbiologists are becoming increasingly aware of the role of *L. monocytogenes* as an infective agent. With the improvement in isolation techniques, for example the use of media containing thallous acetate and nalidixic acid, and the use of cultural characteristics, there should be an apparent increase in the number of diagnosed human cases of listeriosis. However, the picture will not be complete until the epidemiologists insist that *L. monocytogenes* infections are notified.

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