Cairo University
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Advanced studies on *Mycoplasma* spp. Causing problems in commercial vaccines.

AThesis presented
By

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Abstract

The contamination of cell cultures by *Mycoplasma* remains a major problem in cell culture and vaccine manufacture. *Mycoplasma* can produce a virtually unlimited variety of effects in the cultures they infect. These organisms are resistant to most antibiotics commonly employed in cell cultures. Here we provide a concise overview of the current knowledge on: (1) the incidence and sources of *Mycoplasma* contamination in cell cultures, the *Mycoplasma* species most commonly detected in cell cultures, and the effects of *Mycoplasma* on the function and uses of infected cell cultures (2) the various techniques available for the detection of *Mycoplasma* with particular emphasis on the most reliable detection methods and (3) Phylogenic analysis of *Mycoplasma* strains isolated from Local veterinary viral Vaccines. The availability of accurate, sensitive and reliable detection methods and the application of robust and successful elimination methods provide powerful means for overcoming the problem of *Mycoplasma* contamination in vaccines manufacture.

Key words: mycoplasma, contamination, vaccines, cell culture, elimination.

1. INTRODUCTION

Mycoplasma contamination is considered a serious problem not only because their role as a pathogen can cause various diseases in human, animals ,and plants but also it can cause distreous contamination effects on eukaryotic cells that may indicate insufficient care has been taken during vaccine manufacture or quality control.

Mycoplasmas have been nicknamed the “crabgrass” of cell cultures because their infections are persistent, difficult to detect, diagnose, and difficult to cure. The origin of contaminating *Mycoplasma* is in components of the culture medium, particularly serum, or in the flora of the technician's mouth, spread by droplet infection.

Mycoplasma was derived from the Greek words (mykes) for (fungus) and (plasma) for something (formed or molded) (from Wikipedia the free encyclopedia; 2007) that formally belong to taxonomic class Mollicutes. Cell cultures may become infected with a variety of species from the genera *Mycoplasma* and *Acholeplasma* and the possibility of infection by other mollicute genera. (Rottem, S., and M. F. Barile.,1993)

Contrary to other bacteria, *Mycoplasma* grow very slowly, even under optimal conditions. The generation times usually range between one and three hours, but there are also generation times of up to nine hours; in addition, *Mycoplasmas* have a relatively long lag phase. Therefore, it may take more than one week to obtain visible colonies on agar. The location of *Mycoplasma* was assumed to be on the surface of cells. However, recent data have

confirmed its intracellular location using both in-vitro and in-vivo techniques (Kumagai et al., 1971; Shankargouda et al., 2015).

Mycoplasma is the smallest known cell and is about 0.1 µm in diameter. The outer layer is instead, a three layered membrane containing sterols. Diameters of these organisms may range from 0.2-0.3 µm and, due to their plasticity, are able to pass through the pores of a 0.2 micron filter with applied pressure. Because the morphology of *Mycoplasma* is pleomorphic, they occur as two different structural forms during a life cycle: coccoidal, a spherical or spheroidal shape, and filamentous, resembling rods.

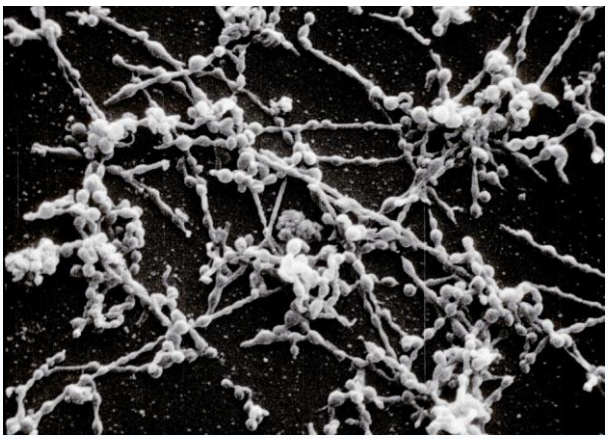


Photo 1 - A common feature is the variability in cell morphology related to the absence of a cell wall. SEM of *Mycoplasma* filaments - Image courtesy of Dr. Michael Gabridge.

Mycoplasmas are a specific and unique species of bacteria - the smallest free-living organism known on the planet. The primary differences between *Mycoplasma* and other bacteria is that bacteria have a solid cell-wall structure and they can grow in the simplest culture media. *Mycoplasma* however, do not have a cell wall, and like a tiny jellyfish with a pliable membrane, can take on many different shapes which make them difficult to identify, even under a high powered electron microscope. *Mycoplasma* can also

be very hard to culture in the laboratory and are often missed as pathogenic causes of diseases for this reason.

Because *Mycoplasma* lacks a cell wall, the organisms are poorly stained, if at all, by bacterial stains. With the exception of *M.hyorhinis*, most *Mycoplasma* can be cultivated using standardized and *Mycoplasma* agar formulations, as well as in broth media, although growth is slow. When grown on agar, the colonies have a “fried-egg” appearance since the colony center grows into the agar and appears denser than the rest of the colony.

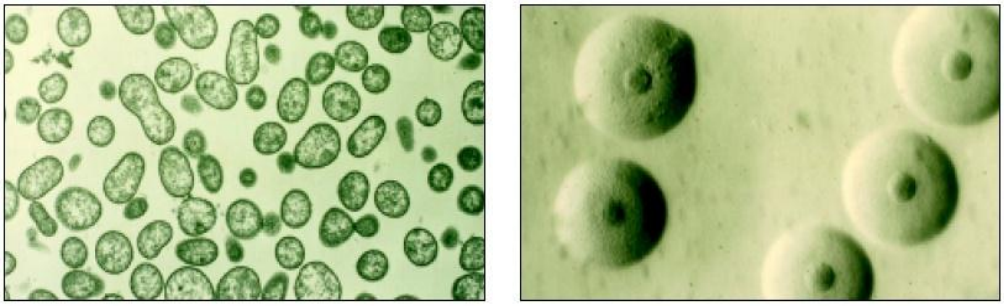


photo 2.3. Viewed under the electron microscope, *Mycoplasma* organisms (above left) may vary in shape due to the absence of a cell wall. Right: Milk samples positive for *Mycoplasma* will produce characteristic “fried egg” shaped colonies when grown on special media. (Dr. Ronald Griffith, Iowa State University, College of Veterinary Medicine, Department of Veterinary Microbiology and Preventive Medicine.)

Mycoplasma is a term for a group of organisms that are classified as neither bacteria nor viruses. They can be parasitic or saprophytic. Several species are pathogenic in humans, including *M. pneumoniae*, which is an important cause of pneumonia and respiratory disorders, and *M. genitalium*, which is believed to be involved in pelvic inflammatory diseases. They are unaffected by antibiotics that target cell wall synthesis, such as penicillin.

Table (1) The Main Human *Mycoplasma* Pathogens
Pathogen / Implicated Disease (*Mycoplasmas* Stealth Pathogens http: //www.rain-tree.com/myco.htm).

Mycoplasma genitalium	Arthritis, chronic nongonococcal urethritis, chronic pelvic inflammatory disease, other urogenital infections and diseases, infertility, AIDS/HIV
Mycoplasma fermentans	Arthritis, Gulf War Syndrome, Fibromyalgia, Chronic Fatigue Syndrome, Lupus, AIDS/HIV, autoimmune diseases, ALS, psoriasis and Scleroderma, Crohn's and IBS, cancer, endocrine disorders, Multiple Sclerosis, diabetes
Mycoplasma salivarium	Arthritis, TMJ disorders, Eye and ear disorders and infections, gingivitis, periodontal diseases including even cavities.
Mycoplasma hominis and Ureaplasma urealyticum	Pelvic inflammatory disease, infertility, non-gonococcal urethritis, vaginitis, cervicitis, amnionitis, pyelonephritis, post-partum septicemia, neonatal pneumonia, neonatal conjunctivitis, Reiter's syndrome, peritonitis, wound infections (C-section), low birth weight infants, and premature rupture of membranes.
Mycoplasma pneumonia	Pneumonia, asthma, upper and lower respiratory diseases, heart diseases, leukemia, Steven-Johnson syndrome, polyarthritis or septic arthritis, CNS disorders and diseases, urinary tract infections, Crohn's and Irritable Bowel Syndrome, Guillain-Barr syndrome, polyradiculitis, encephalitis, and septic meningitis, autoimmune diseases.
Mycoplasma incognitus and Mycoplasma penetrans	AIDS/HIV, urogenital infections and diseases, Autoimmune disorders and diseases
Mycoplasma pirum	Urogenital infections and diseases, AIDS/HIV
Mycoplasma faucium, M. lipophilum and M. buccale	Diseases of the gingival crevices and respiratory tract

Table 2 . *Mycoplasmas* and Associated Animal Diseases (Excluding Poultry) (Carter and Wise, 2004).

Animal	*Mycoplasma/Ureaplasma*	Disease
Cattle	*M.alkalescens*	Mastitis, arthritis
	M.bovigenitalium	Mastitis, arthritis
	M.bovirhinis	Mastitis
	M.bovis	Pneumonia, various infections
	M.bovoculi	Keratconjunctivitis
	M.californicum	Mastitis, arthritis
	M.canadense	Mastitis
	M.dispar	Mastitis, arthritis
	M.mycoids subsp mycoids(SC)[*]	Contagious pleuropneumonia
	U.diversum	Vulvovaginitis,infertility,abortion
Swine	*M.hyopneumoniae*	Enzootic pneumonia
	M.hyorhinis *M.hyosynoviae*	Polyserositis,arthritis Polyartheritis
Sheep	*M.agalactiae*	Contagious agalactia
	M.capricolum subsp. Capricolum	Septicemia, pneumonia, mastitis, arthritis
	M.conjunctivae	Keratconjunctivitis
	M. mycoids subsp mycoids(LC)[*]	Pleuropneumonia Septicemia, arthritis
	M.ovipneumoniae	pneumonia

Goats	*M.agalactiae*	Contagious agalactia
	M.capricolum subsp. Capricolum	Septicemia, pneumonia, mastitis, arthritis
	M.capricolum subsp.capripneumoniae	Contagious bovine pleuropneumonia
	M.conjunctivae	Keratconjunctivitis
	M. mycoids subsp capri	Pleuropneumonia, Septicemia, arthritis
	M. mycoids subsp mycoids(LC)	Pleuropneumonia, Septicemia, arthritis
	M.putrefaciens	mastitis, arthritis
Horses	*M. equigenitalium*	Abortion
	M.felis	Pleuritis
Dogs	*M.canis*	Urogenital tract infection
	M.cynos	Pneumonia
	M.spumans	Arthritis
Cats	*M.felis*	Conjunctivitis
	M.gateae	Arthritis
Mice	*M.neurolyticum*	Conjunctivitis; neurological infection
Rats	*M.pulmonis*	Respiratory infections
	M.arthitidis	Arthritis
	M.pulmonis	Respiratory and genital tract infections

*SC= Small colony ; LC= Large colony

Table(3) Types of Avian *Mycoplasma* :

Species	Main host	Glucose fermentation	Arginine hydrolysis
M.anatis	Duck,goose	+	-
M.anseris	Goose	-	+
M.buteonis	Buzzard	+	-
M.cloacale	Turkey,goose	-	+
M.columbinasale	Pigeon	-	+
M.columbinum	Pigeon	-	+
M.columborale	Pigeon	+	-
M.corogypsi	Vulture	+	-
M.falconis	Falcon	-	+
M.gallinaceum	Chicken,pheasant,partridge	+	-
M.gallinarum	Chicken,turkey	-	+
M.gallisepticum	Chicken,turkey,pheasant,partidge, vulture	+	_
M.gallopavonis	Turkey	+	_
M.glycophilum	Chicken,pheasant,,partidge	+	-
M.gypsis	Vulture	-	+
M.iners	Chicken,turkey,pheasant,partidge	-	+
M.iowae	Chicken,turkey	+	+
M.imitans	Duck,goose,partridge	+	-
M.lipofaciens	Chicken,turkey	+	+
M.melegridis	Chicken,pheasant,partridge	-	+
M.pullorum	Starling(European)	+	-
M.stumi	Chicken,turkey	+	-
M.synoviae		+	-

Adapted from Bradbury,(2001)

The incidence of occurrence of contamination to live vaccines propagated on embryonated chicken eggs was reported by Bankowski (1958),koski et al (1975) and joseph et al. (1988). Where the contamination had become a potential problem for the live vaccines propagated on tissue cultures Clark et al. (1972) and Nicholas et al. (1988).

There's possible contamination of poultry live vaccines with *Mycoplasma* Cernik (1967) were *Mycoplasma* had been isolated from live vaccines by many authors such as Richter(1965) who stated the basic method for testing vaccines against poultry diseses, Clark et al, (1972) isolated *Mycoplasma* antigens from

vaccines propagated on cell culture. koski et al, (1975)identified the *Mycoplasmas* isolated from viral vaccines for veterinary use, Joseph (1988) in Malaysia, isolated *M.gallisepticum* and *M.synoviae* from egg adapted live viral vaccines.

Kojima et al,(1996) in Tokyo detected of *Mycoplasma* in avian live virus vaccines by PCR. Garth Nicolson (2011) He is not just saying that vaccines are contaminated with *Mycoplasma* but is going further and says that we are all being damaged by them and contracting chronic degenerative diseases

There are currently more than 183 species in 8 genera, many of which are pathogenic (The Manual of Clinical Microbiology, 2003). The vast majority of cell culture contaminants belong to only 6 species primarily of human, bovine or porcine origin. Some common organisms that cause cell culture contamination include *M. Hyorhinis* from porcine, *M. Arginini* from bovine, and *M. Orales* and *M. Fermentans* whose natural hosts are humans. Of these six species, *M. orale* and *M. hyorhinis* are the most common historically, accounting for over 50% of all *Mycoplasma* contaminated cultures (Del Giudice and Gardella, 1984; McGarrity et al., 1979; Barile et al., 1973)

Firstly *Mycoplasma* detected in cell cultures in 1956 (Robinson et al., 1956), *Mycoplasma* had become recognized as a major culture contaminant by the 1960s. Published *Mycoplasma* test results for cell lines during this time demonstrated rates of *Mycoplasma* contamination between 57 to 92% (Barile, 1973) . By the 1970s, Barile showed that cultures routinely grown in antibiotics had a 10-fold higher *Mycoplasma* contamination rate (72%) than cultures grown without antibiotics (7%) (Barile, 1973).

The distreous contamination effects of *Mycoplasma* on eukaryotic cells represented in 2 significant problems to the culture of mammalian cells used for research as they can alter every cellular parameter leading to unreliable experiment results and potentially unsafe biological products (vaccines)

First is *Mycoplasma* contaminate cell lines will produce much poorer yield of final cell numbers as they do not grow as high cell density as normal cells.

Second *Mycoplasma* also cannot detected by visual inspection using a normal light microscope and thus can remain unnoticed in cellular cultures for long period of time because *Mycoplasma* are the smallest organism their genome size range from 0.6-1.3 mega base size characterized by lacking cell wall that are capable of self replication and cause various disease in human and animals and plants.

Unless it is specifically tested for, cultures contaminated by *Mycoplasma* often remain undetected since there are no obvious signs of contamination, like the destruction of host cells. Chronic infections usually cause decreased cell proliferation, while acute infections result in total destruction of the cell culture. The following clues may help signal deterioration of a culture affected by *Mycoplasma* contamination:

- Interference with the rate of cell growth
- Changes in cell morphology
- Aberrations in chromosomes
- Altered DNA, RNA, and protein synthesis
- Induced cell transformation

The Most Common Contaminants

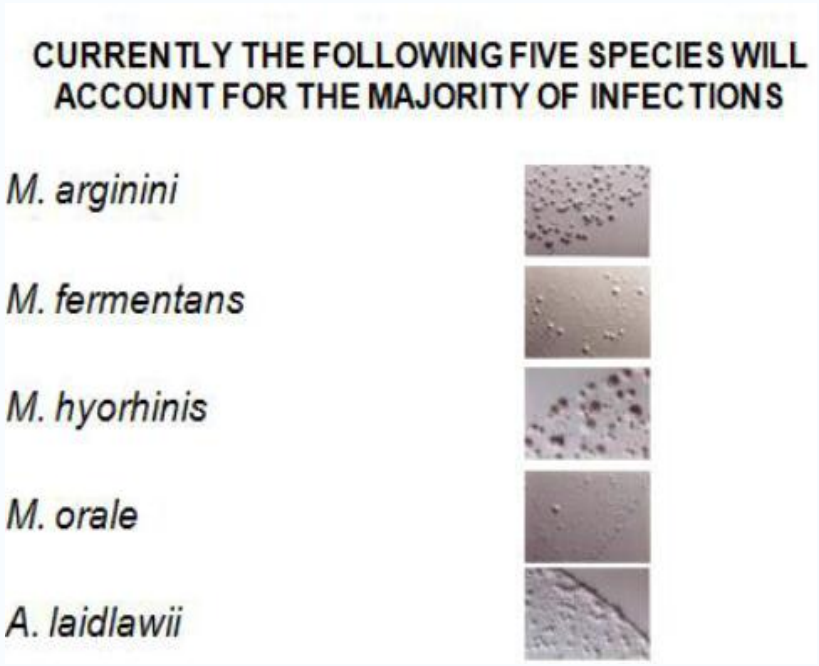


photo 4.*Mycoplasma* spp. Of majority infections for cell cultures (Illustration provided courtesy of *Mycoplasma* Experience)

These five species account for the majority of present day cell culture infections. All have fulfilled the requirements of “survival of the fittest” either by their capacity to adapt to the cell culture environment or by their ubiquity in the environment.

Mycoplasma arginini

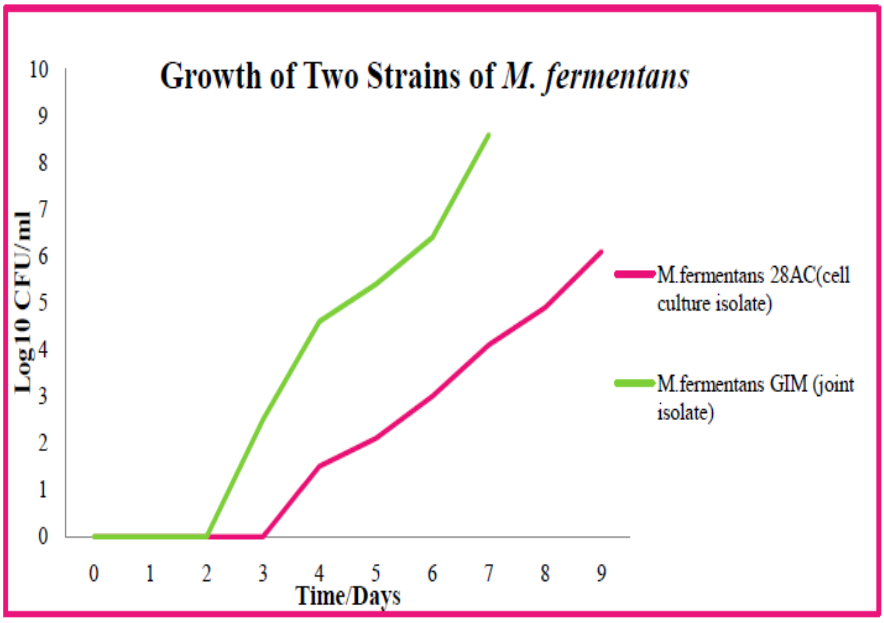
This organism has been isolated from a range of mammalian species including all farm animals. It is one of the two commonest serum contaminants. It is typically very easy to culture, with a rapid growth rate but slower growing, more fragile strains have been isolated from cell cultures. These strains have been associated with antibiotic resistance, particularly to the aminoglycoside antibiotics, neomycin, gentamycin and kanamycin suggesting long term exposure to cell cultures.

M. arginini has been reported to be the most likely species to fail to be detected with the Gibco “Mycotect” system due to low enzyme levels.

Mycoplasma fermentans

This is another organism that may have become adapted to the cell culture environment and thus more difficult to culture. There can be a marked difference in growth rate between strains isolated from the host and cell culture isolates. Slower growing species may establish a low grade infection in more rapidly growing cell lines making them more difficult to detect. It is not known whether the increasing prominence of this organism as a cell culture contaminant is due to changes in the types of cell line being cultivated- which are more susceptible to colonization, or detection failures due to low numbers or adaptation to the cell culture environment.

photo 5.Graph show growth of 2 strains of *M.fermentans*



(Adapted from data kindly supplied by *Mycoplasma* Experience, UK)

Mycoplasma hyorhinis

M. hyorhinis is commonly found in the porcine upper respiratory tract when it is easy to culture, typically giving large colonies on agar after a few days incubation. It is equally common in present day cell cultures where it can be regarded as the most successful “survivor”. Trypsin has often been suggested as the source of *M. hyorhinis* infections as it is sourced from porcine tissue. However this has never been demonstrated and cultures of *M.hyorhinis* inoculated into trypsin solution are rapidly killed although it has been demonstrated experimentally that there is limited survival of the microbial cells if they are aggregated. A more likely original source is bovine serum through abattoir cross contamination. There is evidence to support this proposition as an incidence of a serum lot found to be contaminated with *M.hyorhinis* was reported in the 1970’s. It is likely that this event would occur far less frequently than contamination with the bovine associated species which emphasizes how successful this organism has been at evading detection and spreading through cell cultures worldwide.

M. hyorhinis was the first species to be recognized as having lost the ability to grow on conventional *Mycoplasma* media after adaptation to the cell culture environment. It was realized in the 1970’s that some cell lines were contaminated with strains of *M.hyorhinis* which could not be cultivated. The Hoechst DNA stain method was specifically developed to allow detection of these strains. A small number of laboratories concentrated on developing medium formulations which would support growth of these “non-cultivable” strains.