USE OF ANIMAL FREE COMPONENTS IN THE COMMERCIAL

MANUFACTURE OF VETERINARY CLOSTRIDIAL ANTIGENS

By

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ABSTRACT

The use of animal-origin components and media in the commercial production of clostridial antigens for veterinary vaccine manufacture constitutes an unnecessary and excessive risk to food safety and health. For over 70 years, bacterial strains of the genus *Clostridium* have been used in the manufacture of veterinary and human vaccines to prevent infection and death. The vast majority of these products are made using animal-based components, including brain, heart and liver infusions, meat peptones and casein digests. Over the last twenty years, the threat of Transmissible Spongiform Encephalopathy (TSE) and adventitious oncogenic viral transmission has generated a significant concern about the safety and reliability of animal-based products. While many animal-free options have been developed in recent years, few manufacturers have adapted vaccine production processes to incorporate such media, primarily due to monetary costs, regulatory restrictions, and corporate inertia. Several manufacturers have made progress on the transition from animal-based to plant-based medias to reduce the TSE risk, but the majority of clostridial vaccines manufactured worldwide are still in animal-based media. While the regulatory agencies responsible for overseeing the human pharmaceutical and vaccine manufacturers have been aggressive in requiring the removal of animal-based components from the products, the animal health regulatory bodies have been slower to follow suit. A rapid and aggressive shift in policy is needed to provide the necessary changes to eliminate the risk of TSE from the livestock vaccine industry.

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Chapter 1 – THE CLOSTRIDIA

Ancient Threats in a Modern World

Tetanus, gangrene, and botulism. Throughout history, the diseases caused by bacteria in the genus *Clostridium* have killed, disabled and disfigured countless millions of people. Equally as damaging has been the loss of livestock upon which our diets depend. It has only been in the last century with the advent of vaccines and modern medicine that the scourge of these terrifying threats has diminished to near imperceptible memories. Yet underlying the seemingly miraculous cures brought about by such vaccines and treatments lie hidden dangers that only in the last few decades have been recognized.

The cause of this new threat is the way the vaccines are manufactured, including the media and components used to make them. For well over 100 years, the most common basal component in clostridial growth media has been bovine sourced meat and organs. For the clostridia, there are few more effective substrates to live and grow in than an oxygen-deprived, sulfide-laced stew of boiled beef muscle, brains, livers and hearts. The bacteria grow fast and efficiently, and pump out deadly toxins in large quantities in this enriched medium. In the contained setting of a modern veterinary vaccine manufacturing facility, these toxins and antigenic cellular components are

concentrated, purified, refined and formulated into life-saving vaccines and medicines that have saved millions of lives and billions of dollars worldwide.

The first recorded instances of clostridial disease date from the Greek physician Hippocrates around 400 B.C., when he described a disease that has been diagnosed as gas gangrene, caused by the bacterium *Clostridium histolyticum*. Tetanus was first definitively described in Sir Charles Bell's *Essays on the Anatomy and Philosophy of Expression* in 1824 (Bell, 1824). However, recognition of the disease states did not provide a direct diagnosis of the causative organisms. It wasn't until the 1860s when Louis Pasteur described a microbe that was capable of growing without air, which he dubbed *Vibrio butyrique*, that the first identified member of the genus *Clostridium* was recognized (Bahl & Dèurre, 2001). He also coined the term "anaerobic" to describe life that could survive without free oxygen.

The first clostridia to be isolated in pure culture were *C. tetani* and *C. butyricum* in the late 1890s, followed by isolation of *C. perfringens* a few years later. Over the course of the following century, over one hundred different clostridial species have been isolated and described, many of which have been put to use in the biomedical and biotechnological industries. However, it has always been the disease-causing species that have received the greatest attention in the public eye. The most potent toxins in the world are made by clostridia – tetanus and botulinum toxins. With a median lethal dose (LD_{50}) of less than 1 ng/kg, infection with only a few spores of either of these bacteria is often enough to cause death to unprotected animals. And while these are the two most

potent toxins in the clostridial genus, they are far from the only ones. The enteric and histotoxic strains generate as much damage and death from diseases such as gangrene, blackleg, enterotoxemia, and others (Smith, 1968).

Farm fields provide an inviting and rich environment for clostridial bacteria to live in. There are abundant food sources, from the feed and animal waste that is spilled liberally on the ground, to the opportunistic infections that can be inflicted upon the livestock roaming the farm. If times get difficult or threatening to the bacteria, they form hardy spores, their own protective survival mechanism. Clostridial spores are extremely resistant to harsh treatments, such as heat, chemicals and radiation, so they will survive exposures and cleaning regimes that remove vegetative bacterial cells. When more compatible circumstances arrive, the spore can rapidly germinate into a replicating vegetative cell which can cause disease and loss of livestock. (Bahl & Dèurre, 2001)

The Diseases They Cause

A frightening list of debilitating, disfiguring and deadly diseases are caused by the clostridia. In humans, the most well known are the neurotoxigenic pathogens, *C. botulinum* and *C. tetani*. Although these are historically the most well known, some lesser known cousins are beginning to make headlines around the world. *Clostridium difficile* has become one of the most dangerous and common nosocomial infections

worldwide, affecting 13 people per 1000 hospitalizations in the United States alone (Goldstein, 2008). The toxins produced by clostridial species are some of the most lethal known, with the neurotoxins from botulinum and tetanus having a human LD_{50} of less than 1 ng/kg (Gill, 1982).

The threat of clostridial infection in farmed animals is even greater. Not only are the neurotoxic organisms a serious problem, but wound infections, hepatic infections and kidney diseases all can create a tremendous economic loss. An animal infected with any one of these diseases is almost always considered a total loss to the producer, with mortality rates of well over 90-95% for nearly all clostridial infections. Because clostridia can survive as spores in exposed soils, stagnant waters and manure, the potential risk of contamination of herd animals is very high.

Skin wounds can be infected with a number of different species, such as *C. novyi*, *C. perfringens* or *C. septicum.* These gangrenous infections, if untreated, will rapidly spread into the musculature of the animal, causing extreme tissue necrosis and eventually death. Other diseases such as redwater, enterotoxicemia, blackleg and tetanus maim and kill many more livestock.

The ubiquitous distribution of clostridial strains in the environment gives rise to a very impressive list of livestock diseases, affecting nearly all species. Cattle, horses, sheep, poultry and swine all are affected by clostridial diseases, with the most prevalent arising in cattle and sheep (Table 1).

It is nearly impossible to avoid the *clostridia* when raising agricultural livestock. Thus, the only way to prevent significant losses to these diseases is through aggressive and prophylactic vaccination. Fortunately, the vaccines that have been developed are extremely efficacious, readily available and very inexpensive.

Table 1: Clostridial Infectious Diseases of Livestock (Sippel, 1972)

Disease	Species affected	Clostridial Species	Predisposing causes	Features and comments
Black disease (infectious necrotic hepatitis)	Sheep mainly. Cattle rarely.	Cl. Novyii	Damage to liver by young migrating liver fluke	Sudden death, particularly in sheep. Plug of yellowish dead tissue in the liver usually on the surface but occasionally deeply embedded, with signs of liver fluke damage. Rapid decomposition of the carcass.
Blackleg	Cattle, sheep	Cl. Chauvoei	Damage to muscles, such as bruising following yarding. In sheep blackleg tends to follow injury, such as at vaccination, shearing, castration	Sudden death. Affected animals are usually in good condition. May be swelling of a leg, the leg may crackle when touched. Rapid decomposition and bloating of the carcass. The carcass should be disposed of to prevent infection of other animals.
Necrotic enteritis	Cattle, poultry	C. perfringens type C	Unknown at present	Bloody diarrhea, lethargy, dehydration. Death in over 95% of cases
Redwater Disease (Bacillary Hemoglobinuria)	Cattle	C. hemolyticum	Damage from liver flukes or other causes	Death is extremely rapid, occurring in 2-3 hours in young animals, and up to 24 hours in older animals, with convulsions prior to death. Rapid carcass decomposition gives rise to 'pulpy kidneys'. Usually seen as outbreaks in sheep
Malignant oedema and Gas gangrene	Sheep mainly, also cattle and goats.	Cl. Septicum, Cl. Perfringens (type A)	Deep wounds such as dog bites, crow attacks and lambing injuries.	Soft swelling followed by a discharge from the wound. Death usually occurs after 1-2 days. Treatment can be attempted through the use of antibiotics and cleaning of the wound.
Enterotoxaemia (pulpy kidney)	Sheep, cattle and goats, particularly young animals	Cl. Perfringens (type C/D), Cl. Sordellii,	High levels of starchy food in the diet and slowing of gut movement.	Death is extremely rapid, occurring in 2-3 hours in young animals, and up to 24 hours in older animals, with convulsions prior to death. Rapid carcass decomposition gives rise to 'pulpy kidneys'. Usually seen as outbreaks in sheep, and the death of just a couple of animals in other species.
Tetanus (lockjaw)	Horses and pigs most susceptible. Cattle, sheep and goats	Cl. Tetani	In sheep, use of rubber rings for marking; dog bites and shearing wounds. In horses, nail pricks in hoof. In cattle after calving. In pigs after castration. Any deep penetrating wounds.	Signs appear 3-10 days after injury in lambs, and longer in other animals. Clinical signs include general body stiffness and muscular spasms, sensitivity to sound and movement, third eyelid protrusion, and restricted jaw movement. Most cases die within 3-4 days

A New Threat to the Food Supply

By vaccinating livestock against the most common clostridial diseases, farmers worldwide have the ability to stem the loss of profit nearly 100% for a very small financial cost. But this small cost comes with the potential for a much greater risk in the future. In 1986, the first known outbreak of a transmissible form of Bovine Spongiform Encephalopathy (BSE) occurred. The number of infected cattle increased for nearly a decade, with no clear medical explanation of how the disease was caused or transmitted. Finally identified as being caused by a prion, the infections were slowly contained by massive culling and quarantine efforts that devastated the British cattle industry. Although prion-related diseases have been known to affect humans, either through heredity (Creutzfelt-Jacob disease) or cannibalism (Kuru), there was no indication that the species barrier between humans and livestock had ever been broken. In 1996, a new and frightening development occurred, variant Creutzfeldt-Jakob disease (vCJD). The species barrier into humans had been crossed. Prions from sheep and cattle were shown to cause the disease in humans, meaning that long-term exposure to diseased animal products could also infect people.

One of the greatest concerns with identifying and tracking prion-based infections and the spread of disease through a herd is the lack of any test for the presence of prions short of sacrificing the animal. The only currently accepted and definitive test for a TSE disease is a brain necropsy to identify lesions and plaques in

the brain. Although several *in vitro* test methodologies have been investigated in the last 10 years, none have yet proven reliable or accurate enough for any commercial use. (Brown, 2005; Supattapone, Geoghegan, & Rees, 2006)

While many raw materials suppliers began reducing and/or eliminating animal based components from their product lines for human vaccine preparation, there was less pressure on the veterinary vaccine industry to make a similar change. The financial and regulatory costs were deemed too great, and without focused government pressure there was little incentive to force a switch.

The Cost of Combating the Clostridial Threat

Modern vaccine preparation methods not only refine and concentrate the clostridial antigens, but they also have the potential to concentrate other very dangerous components that come from the meats and organs that form the media. Prions and host-animal antigenic proteins are all retained in the final product. These unwanted contaminants can cause problems ranging from painful localized reactions at the injection site, a threat to the health of all animals who receive one or more of these vaccines, to an infection risk to every person who consumes the vaccinated animal.

It has been estimated that the risk of contracting a prion-based disease from tetanus vaccination is less than 1 in 5 billion injections (Prusiner, 2004). This is a very small risk, translating to one potential infection in 50 years of vaccination.

Unfortunately, similar risk-assessment is not available for the veterinary vaccinations, but a review of the numbers shows the potential risks can rise to be significant. There are over 96 million head of cattle in the United States, and more than 91% are immunized against clostridial diseases (USDA-APHIS, 2008a). These vaccinations are most often given as a multiplex dose, with a single inoculation containing fractions to protect against up to seven different clostridial strains, and often requiring multiple injections over the lifetime of the animal to confer immunity.

Without vaccination, an animal that contracts a clostridial disease will almost certainly die. Current clostridial vaccines are inexpensive for the consumer to purchase, which restricts the manufacturer's return on investment to only a fraction of a cent per dose. In 2007, the cost to vaccinate a 100-head herd of cattle with a 7-way clostridial vaccine cost less than $25.00 (USDA-APHIS, 2008a). While such a small cost to the farm is considered as a great benefit, the effect on the manufacturer of the vaccine is the opposite because of the small fraction of profit that is made on each dose.

That slim profit margin for the producer means that there is little direct financial incentive to innovate and upgrade a standard process that effectively and cheaply generates the product. Government regulations, which are necessary to ensure the safety, stability and consistency of the vaccines, add to this pressure to resist change. It has been estimated that the costs involved in making a radical media change could be greater than one million dollars per antigen, per vaccine presentation. This is because of the testing, evaluation and regulatory hurdles that must be overcome. Although some

of this cost could be passed on to the consumer, not all of it would be and the remainder would have to be absorbed wholesale by the manufacturer. With most vaccine manufacturers producing between five and seven separate strains and combining them into dozens of multivalent vaccine presentations, the financial pressure to not change is tremendous.

Although there hasn't been a major shift away from meat-based media to date, there is a growing sentiment both in the industry and within the government regulatory commissions to change. Pressure to change to animal-free media and components is growing, and in many areas of the world there are increasingly strict regulations and/or bans on the use of sera and animal products in the manufacture of new vaccines and products. It is only a matter of time before these restrictions and requirements are extended to established products in the United States, which will result in a need for a viable alternative media to replace the firmly entrenched processes now used.

Chapter 2 – CLOSTRIDIAL VACCINES

Current Clostridial Vaccine Manufacturers

There are only six licensed veterinary clostridial vaccine manufacturers in the United States (Table 2). Unlike most of them, United Vaccines produces only a single clostridial bacterial vaccine for protection against *C. botulinum* intoxication. The rest produce numerous vaccines in various formulations with up to seven separate clostridial strains in a single presentation. With so few makers of these critical veterinary vaccines, it is worrisome that there has been so little effort or regulatory pressure to move the production to animal-free components.

Many of these manufacturers have sibling companies that produce human vaccines and biological products. The FDA has been far more stringent and forceful in the push towards an animal-free pharmaceutical mentality, leaving the veterinary sector lagging far behind in the safety and modernity of their processes. This gap is far smaller in the European Union regulated nations, where both human and veterinary biologics are controlled by a single agency. Many of the veterinary manufacturers either sell products or have facilities located in those European countries, so those corporations are more proactive in making an effort to change their processes currently.

Two manufacturers have licensed clostridal products currently being made with animal-free media, Intervet and Pfizer. However, even in those two cases not

TABLE 2: USDA-Licensed Veterinary Clostridial Vaccine Manufacturers

(USDA-APHIS, 2008b)

Company	***Location***	***Clostridium species produced***	***# of vaccines***	***Current Formulation[1]***	***Strains in Highest Combination***
Boehringer Ingelheim Vetmedica	St. Joseph, MO	Chauvoei, Septicum, Haemolyticum, Novyi, Sordellii, Perfringens Types C & D, Tetanus Toxoid	11	CMB, MM	7
Colorado Serum Company	Denver, CO	Chauvoei, Septicum, Novyi, Sordellii, Perfringens Types C & D	19	CMB, MM	7
Intervet, Inc.[2]	Millsboro, DE	Chauvoei, Septicum, Haemolyticum, Novyi, Sordellii, Perfringens Types C & D	20	CMB, MM, AF	7
Novartis Animal Health US, Inc.	Larchwood, IA	Chauvoei, Septicum, Novyi, Sordellii, Perfringens Types A, C & D	13	CMB, MM	7
Pfizer Inc.	Lincoln, NE	Chauvoei, Septicum, Haemolyticum, Novyi, Sordellii, Perfringens Types C & D	8	CMB, MM, AF	7
Schering-Plough Animal Health Corp.[2]	Omaha, NE	Chauvoei, Septicum, Haemolyticum, Novyi, Sordellii, Perfringens Types A, C & D	13	CMB, MM	7
United Vaccines, Inc.	Madison, WI	Botulinum Type C Bacterin	7	CMB, MM	1

[1] CMB = chopped meat broth, MM = Modified Mueller, AF = Animal-Free

[2]Although Schering-Plough acquired Intervet in 2007, both product lines are still produced separately.

all of the clostridial antigens made by the companies use animal-free media. The majority of products are still produced in either chopped meat broth or Modified-Mueller media, meaning that even the two companies leading the way in the switch to animal-free clostridial products are still far from transitioning their entire portfolios over.

Primordial Ooze and Other Growth Media

Most veterinary clostridial vaccines are made using one of only a few choices in media preparation (Table 3), either a chopped-meat broth (CMB) or a peptone-based complex media (modified Muller) (Feeney, Mueller, & Miller, 1943; Mueller & Miller, 1942) (Latham et al., 1962). While most manufacturers have adapted these recipes in recent decades to further optimize the antigenicity of their particular strain of organism, only a small percentage have succeeded in shifting any of their clostridial vaccines to non-animal based media. This reliance on large quantities of beef products for production, in some cases including beef brain and heart infusions, increasingly raises the spectre of BSE being transmitted through the vaccination of healthy herds.

The most common medium for manufacturing clostridial vaccines is a basic concoction of boiled ground beef or liver combined with several salts. While highly effective for promoting growth and toxin production of clostridial strains, there is a lack of consistency and reproducibility due to the nature of the product. Lot to lot

Table 3: Standard Industrial Recipes for Clostridial Production

		1943	1954	1962	1985	2007
		Chopped Meat Broth[1]	**Miller-Muller[2]**	**Latham[3]**	**Reinforced Clostridial Broth[4]**	**ISPAH Soy Media[5]**
Protein	Ground Meat	500 g	0 g	0 g	0 g	0 g
	Beef Extract	0 g	0 g	0 g	10 g	0 g
	Casein Digest	30 g	22.5 g	25 g	0 g	0 g
	Tryptose	0 g	0 g	0 g	10 g	0 g
	Beef Heart Infusion	0 g	50 ml	0	0 g	0 g
	Soy Peptone	0 g	0 g	0 g	0 g	45 g
	Yeast Extract	5 g	0 g	0 g	3 g	0 g
Sugar	Glucose	10 g	11 g	8 g	5 g	10 g
Salts and Minerals	NaCl	0 g	2.5 g	2.5 g	5 g	5 g
	Sodium Acetate	0 g	0 g	0 g	3 g	5 g
	Sodium Phosphate	0 g	2 g	0	0 g	0 g
	Potassium Phosphate	5 g	0.15 g	0	0 g	0 g
	Magnesium Sulfate	0 g	0.15 g	0.1 g	0 g	0 g
	Cysteine	0.5 g	0.25 g	0.125 g	0.5 g	0.5 g
	Tyrosine	0 g	0.5 g	0	0 g	0 g
	Calcium Pantothenate	0 g	1 mg	1 mg	0 g	0 g
	Uracil	0 g	2.5 mg	1.25 mg	0 g	0 g
	Nicotinic Acid	0 g	0	0.25 mg	0 g	0 g
	Thiamine	0 g	0.25 mg	0.25 mg	0 g	0 g
	Riboflavin	0 g	0.25 mg	0.25 mg	0 g	0 g
	Pyridoxine	0 g	0.25 mg	0.25 mg	0 g	0 g
	Biotin	0 g	2.5 µg	2.5 µg	0 g	0 g
	Vitamin B12	0 g	0	0.05 mg	0 g	0 g
Iron	Reduced Iron Powder	0 g	0.5 g	0	0 g	0 g
	FeCl3 • 6H2O	0 g	0	32 mg	0 g	50 mg

1 – (Mueller et al., 1943)
2 –(Mueller & Miller, 1954)
3 – (Latham et al., 1962)
4 – (MacFaddin, 1985)
5 – (Unpublished Research, Intervet-Schering Plough Animal Health)

variations in the meat, ranging from varying fat and protein levels to age and condition of the cattle from which the meat was rendered. Added to that inconsistency is the increasing challenge of TSE diseases, as well as the potential for antigenic incompatibility with vaccinated animals results in a major challenge to the veterinary vaccine industry. This remains the most commonly used medium for clostridial vaccine antigen production in the world.

Several clostridial vaccines are made with a more defined medium based on the modified Mueller method , but even in these products significant residues of beef proteins, including the possibility of TSE infectious prions remain (Prusiner, 2004). And even these more defined media retain the potential for adverse anaphylactic reactions to non-host species proteins. Because almost all animal food allergens have homologues in related species, these are far more likely to cause an anaphylactic reaction than an evolutionarily more distant species (Jenkins, Breiteneder, & Mills, 2007). Anaphylactic reactions can range from minor site reactions, such as swelling and tenderness at the injection site, to systemic effects of fever, hemorrhaging, and even death. While the percentage of vaccines made using this modified medium is growing, it still contains a very significant portion of animal-based components (peptones and caseins).

Manufacturing Methods for Clostridial Vaccines

Because of the limited number of manufacturers in the United States, there is a limited repertoire of manufacturing methodologies and vaccine presentations available for clostridial vaccines. A few are highly refined and comparable to the quality and consistency of human vaccine presentations of single-protein purified antigens. But most are produced as a much cruder and cheaper product.

The clostridial vaccines are produced by all veterinary vaccine manufacturers in nearly the same manner, with only minor modifications in media formulation and processing that are company/site specific. The frozen seed stocks are subcultured into a volume of the standard medium, and passaged until a sufficient volume is generated to inoculate a large fermentor – typically 2500L or greater. The fermentor is inoculated with the seed culture and monitored. In some cases, additional sugars are added to enhance growth during the incubation. After reaching the desired harvest criteria, the entire batch is inactivated with formaldehyde. This kills the vegetative cells and any spores that are produced, as well as inactivating the toxigenic proteins to produce toxoids. After a specified period of retention and testing, the bulk product is then processed.

The simplest method for processing a bulk antigen into a vaccine presentation is simply to pass the whole culture across a low molecular weight tangential flow filtration unit in order to remove the majority of fluid volume while retaining all of the cells, debris and immunnogenic proteins in the retentate. This whole-cell

concentrate is then supplemented with an adjuvant and excipients to increase stability and immunogenicity, followed directly by formulation into the final product.

A slightly more refined method of processing involves using one of the available methods to remove the whole cells prior to low molecular weight tangential flow filtration to reduce the product volume. While this procedure eliminates the large cells and majority of the debris, it still allows the majority all of the free proteins and nucleic acids to pass into the final concentrated product, including prion proteins. Without extensive and expensive downstream processing like column chromatography or differential saltation precipitation, it is almost impossible to remove contaminating adventitious agents and prions from vaccine concentrates.

Approximately half of all clostridial products have the cells and cell-debris removed prior to final processing by either continuous flow centrifugation or tangential flow filtration using micron-scale filters. In all products, the final filtration step is processing over tangential flow membranes with a molecular cutoff of between 10kD and 50kD. The final concentration step typically results in a reduction of volume of over 25-60 times (2500L reduced to between 40-100L). The concentrated bulk is mixed with other concentrated immunogens and excipients to make up the final dosed product. While some vaccines are monovalent (containing a single immunogen), most for cattle, sheep and swine are polyvalent, containing up to 10 different immunogens. This allows for a more cost effective and efficient means of vaccination for the farmer, but it also increases the overall

percentage per dose of concentrated beef products – and potentially prions and other unwanted contaminants. While newer reformulations of some vaccines use as little as two milliliters in a dose for a 1000 pound cow, most vaccinations are between five and ten milliliters per dose. Depending on the number of immunogens in the vaccine preparation, over 80% of that volume may be concentrated antigen (USDA-APHIS, 2008b) and all of the residual material from the production medium and cell debris.

In clostridial vaccine preparation, retaining the greatest percentage of immunogenicity in the final product is critical to ensure an efficacious dose in as small a volume as possible, while reducing the cost per dose. Because the toxigenic proteins in most clostridial strains range from 60kD to 180kD, the most common processing filtration is done with 10kD nominal filters. These provide efficient and effective concentration of the desired product, while allowing a rapid dewatering of the bulk to maximally reduce product volume. Unfortunately, this also carries the potential for retention of other components and potentially antigenic fragments that are larger than the nominal size cutoff.

CHAPTER 3 - NEW RISKS IN OLD TECHNOLOGY

The Start of a New Epidemic

In 1986, several dozen cattle in Great Britain became ill with a new disease, and over the course of the following decade this evolved into a devastating epidemic that nearly wiped out the entire British beef and dairy cattle industry. Bovine Spongiform Encepalopathy (BSE) would have been worrisome enough if it had restricted itself to just cattle. But just a few years after the initial outbreak in cattle, several humans contracted a frighteningly similar disease called variant Creutzfeld-Jacob Disease (vCJD) (Prusiner, 2004).

These diseases are unusual. The infectious agent wasn't a virus, bacterium or fungus, and it didn't even have nucleic acid. The causative agent was a misfolded protein called PrP, which accumulated into amyloid plaques within the brain (McKinley, Bolton, & Prusiner, 1983). These plaques caused lesions in the brain, eventually turning the normal brain into a spongy, debilitated mass of tissue. The animals and people affected slowly lost motor, balance and cognitive function, which inevitably lead to death.

Although this type of disease hadn't been documented in cattle previously, a similar type of disease was well known to the sheep farmers – scrapie. It was known that scrapie could be transmitted by feeding rendered animal carcasses to sheep, and the same transmissibility was shown to occur in both cattle and humans.

Prion proteins fall into the size range retained by the typical processing parameters of vaccine manufacturers. The typical prion exists as a dimer of approximately 55kD (Prusiner, 2004). In a typical batch production lot of vaccine made with chopped meat broth, over 15% of the final volume is concentrated beef broth and extract. If a prion-infected carcass is used in the production of the antigen, then it is highly likely that the final bulk product will contain at least some measurable amount of prion protein.

Although the meat and peptone products are fully autoclaved before inoculation, and the final culture harvest is inactivated with moderate concentrations of formaldehyde (0.5-1.0% vol/vol), there is still a concern over potential prion contamination. Prions remain infectious in conditions that render nearly all other infectious particles and organisms inactive. The protein amyloids can survive autoclaving at 132°C for over 240 minutes and exposure to formaldehyde concentrations up to 10% for over one hour (Giles et al., 2008). The denaturing conditions necessary to render prions inactive would result in a complete loss of immunogenic activity in the clostridial antigens being processed. And because prions form aggregate amyloids of random size distribution, filtration to eliminate the contamination is not effective (Prusiner, 2004).

Holes In The Defenses

While the USDA has restricted the importation of bovine products from nations that have endemic BSE and screening and testing programs are in place at processing plants within the US, the testing is not 100% effective. There have been numerous failures over the last several years in which BSE infected cattle have been imported and rendered at plants within the US. As recently as 2006, BSE infected cattle have been identified by the USDA within USA feedstock and dairy livestock (USDA-APHIS, 2006).

In 2004, 2005 and 2006, BSE infected cattle were discovered by the USDA screening and monitoring procedures. In the 2004 case, the infected cattle were originally imported through Canada to the state of Washington. In the subsequent investigation, the USDA was unable to account for all the index case's cohort animals which were at high risk for infection. Out of the 25 animals classified as "higher risk" of having BSE, only 14 were definitively identified and humanely culled, along with 230 other cattle in the suspect herds. The index cow also had two offspring, which had been sold at auction to other operations. After determining the identity and location of those two cattle, the entire herds were humanely euthanized - a total of 579 animals (USDA-APHIS, 2004). In 2005, another identified case of BSE-infected cattle occurred in Texas. Unlike in the 2004 Washington incident, the offspring of this cow could not be positively traced in all instances. Because of the volume of cattle moving through the slaughter and processing system and the lack of

identifiable records on the originating farm, several cohorts and offspring were unaccounted for even after extensive investigation (USDA-APHIS, 2005). Less than a year later, another confirmed infection was identified in Alabama. Like the Texas case, the lack of farm records hindered the tracing of all progeny and cohorts. The herd of origin was never identified, nor were all of the offspring of the infected cow (USDA-APHIS, 2006).

The European Union, one of the regions hardest hit by BSE infections, issued a report in 2004 (GBR, 2004) which determined that the current risk of BSE infectivity in the USA was "likely but not confirmed that domestic cattle are (clinically or pre-clinically) infected with BSE-agent." In their summary, the European Food Safety Authority determined that the risk to US cattle was not just from suspect imports arriving from at-risk nations, but also an internal threat due to the lack of an integrated, national animal identification system and a passive surveillance system, with only a small portion of active oversight in the highest "at-risk" areas. Based on the discovery of additional BSE infected cattle in the years following the European report, and the failure to trace all potential infective progeny and cohorts in those several cases demonstrates risk to US food safety, and the need to reduce any risk and challenge from BSE.

There is little data available on the potential for iatrogenic transmission of prions through vaccination vectors. Because of this dearth of empirical data, risks must be assessed through similar evaluations of other transmission means such as

blood and plasma transfusions in humans. This is not ideal, however it is the only data available at present to compare risk of infections.

From 2003 to 2007, there have been four documented cases of vCJD which were contracted from infected blood transfusion products (Turner & Ludlam, 2009). From the data presented, the risk for infection from transfusion is low, but still significant because there is no currently available screening test to detect prion proteins in the blood supply (Liras, 2008). While human-human transmission through plasma and blood transfusions will most likely remain low due to increased scrutiny and monitoring of the donor blood supply, the risk of livestock infection from vaccinations containing high concentrations of bovine-derived components remains potentially significant.

For over 20 years, the only definitive confirmation for BSE infection has been a post-mortem dissection and examination of the brain for lesions and mis-folded prion proteins. Although several companies and research groups are actively working on an in-vitro live animal assay for prion infection, none have succeeded. Because there is no available test for prion proteins in live animals, it falls to the farmers and livestock processors to maintain vigilance and care in reporting suspect abnormal behavior in their animals.

Chapter 4 – ANIMAL-FREE COMPONENTS

Plant Based Animal Free Components

In the past, the availability of animal free media components was limited to a few plant based peptones or completely chemically-defined artificial media. Within the last few decades, this limited repertoire has been enhanced with a great number of new manufacturers and products. From the original soy-based plant peptones, there is now a tremendous diversity of products available, made from soy, potato, wheat, corn, pea, cottonseed and more.

While these basal ingredients are considered "animal-free", there is often some component of animal origin used during the processing and manufacture of the products. Depending on the federal and/or international regulations that govern the labeling of media products, these additional animal-origin components may or may not be listed in the product literature. Some of these additions are used in enzymatic treatments with extracts made from pancreatic and other organ lysates, to break down the protein and starch components into usable peptones and sugars. These animal extracts are a minimal fraction of the total weight of the final product, but this can still affect the claim of "animal free" from the product.

The alternative to using animal based enzymes to break down the proteins and starches is hydrolysis, which is effective but can be more expensive. With increased pressure on manufacturers and a rising demand for 100% animal-free

products, these costs are steadily decreasing. In 2009, Becton-Dickinson opened the world's first fully animal-free component manufacturing facility in Miami, Florida (Biosciences, 2009). This facility will allow the production of 100% animal-free components under the strictest FDA and EU manufacturing standards to ensure zero cross-contamination of products and defined traceability of all raw materials back to the tertiary level. However, these new manufactured materials come with a high cost, which puts veterinary biological production at a distinct disadvantage. While the prices of human pharmaceuticals and biologicals can rise much more readily with an increase in cost in raw materials, veterinary manufacturers are not so fortunate. Adding in the higher costs of raw materials will, at least in the short term, result in a lower return on investment per dose. This, along with the increased costs associated with regulatory testing and validation of new processes, reduces the overall incentive for manufacturers to convert to a fully animal-free process.

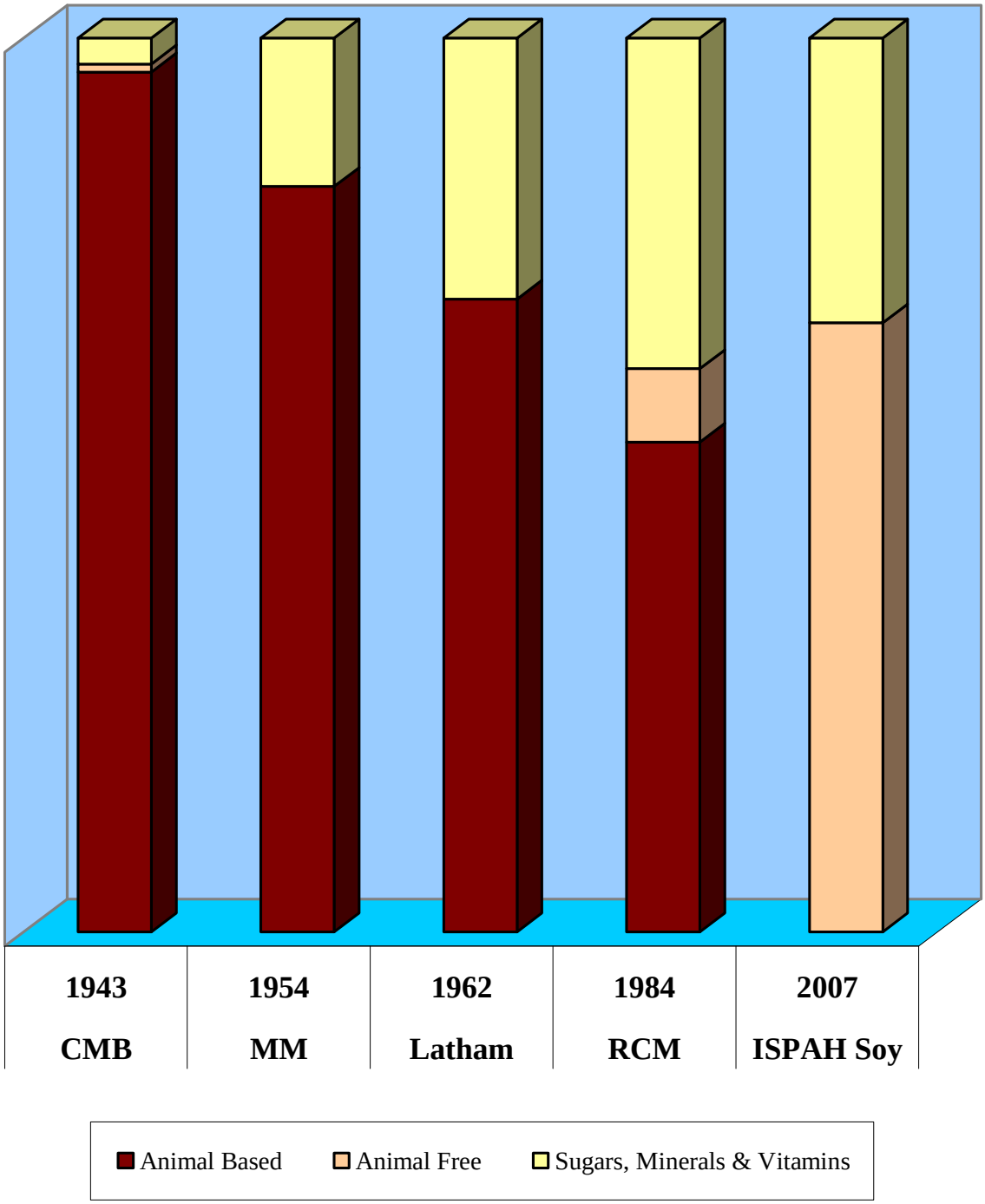
Animal free media have been used in the past to manufacture some clostridial species. As early as 1971, it was shown that *C. perfringens* could be grown successfully in a medium containing soy proteins (Busta & Schroder, 1971). In the last decade, there has been an emphasis in the human biological pharmaceutical industry to use soy-based media for manufacturing *C. tetani* and *C. botulinum*. Because tetanus toxoid is a highly purified and concentrated antigen that is administered to humans, there is a much greater awareness and concern with potential contaminates like BSE. In 2007, a paper by Fang *et al*. outlined a soy-based medium that was successfully used to manufacture tetanus toxoid (Demain,

George, Kole, Gerson, & Fang, 2007). Based on these studies, it is clear that clostridial strains can be readily grown in media free from animal products.

In 2007, researchers at Intervet-Schering Plough Animal Health (ISPAH) developed a non-animal based medium formula using a soy peptone base which allowed strong growth and toxin production in several clostridial strains. This new medium formulation eliminates all sources of animal derived components, however it is not in full production for all clostridial vaccine strains. Continued efforts have been made in the last two years to improve the media and modify the formula to enhance growth and toxin production of all commercial vaccine strains.

Over the decades, from the first clostridial vaccine manufacturing processes of the 1940's up through today, there has been a minor effort to reduce the percentage of animal-based products in the production media. From the original chopped meat broths, which were composed of nearly 100% animal products, through the 1960's and up to the 1990's with modified component media slowly upgraded and improved, there has remained a reliance on animal based components. It has only been in the last few years that the practical, full-scale production of completely animal-free media has been emphasized. (Figure 1) These animal-free products only represent a few percent of the total vaccine production worldwide, with the vast majority still produced using animal-based components, with nearly half of those products made in CMB, the medium most at risk for transmitting BSE (Constance, 2009).

Figure 1: Reduction of Animal-Based Components in Clostridial Media



CMB – (Mueller, Schoenbach, Jezukawicz, & Miller, 1943), MM –(Mueller & Miller, 1954), Latham – (Latham, Bent, & Levine, 1962), RCM – (MacFaddin, 1985), ISPAH Soy – (Unpublished Research, Intervet-Schering Plough Animal Health)

Advantages and Benefits of Switching To Animal-Free Media

The most obvious benefit in converting to an animal-free media base for clostridial vaccine production is the immediate end to any risk of prion-transmissible disease. With no animal source in any of the products, the risk of post-vaccination infection, and the potential for subsequent introduction into the human food supply, is eliminated.

When animal-based products are used in the manufacture of a vaccine, the possibility exists for an anaphylactic reaction to antigenic peptides that remain in the preparation from the source animal (Ellenberg, 2001). In the case of cattle receiving an injection made from bovine products, this is not a major concern. But in the case of sheep, poultry or swine, these bovine antigenic peptides can result in significant losses of market weight (Cooper & Jull, 1966). These reactions can range from large and painful site reactions to death. The site reactions result in damage to the animal's meat, which must be removed prior to processing (Stokka, Edwards, Spire, Brandt, & Smith, 1994). This can cause a loss of several pounds of meat, and a concomitant loss of revenue for the farmer. In extreme cases, the reaction has been strong enough to sicken the animals enough to reduce final weight by 10-20%, which represents a significant financial loss. In rare cases, there is enough of an anaphylactic reaction to kill the animal, resulting in a 100% loss of investment. One significant advantage of using plant-based media is that there is a far smaller incidence of immunogenic anaphylactic reaction in the receiving animal, so the

damage and loss is reduced considerably (Jenkins et al., 2007; Wells, Pass, & Eyre, 1974).

Although there is a significant up-front cost associated with moving production to animal-free media due to testing, evaluation and regulatory compliance issues, the long term benefits are tremendous. Not only is the cost of the raw materials often significantly lower, but the labor, equipment and facility expenses are also reduced less. In addition, there is the potential to more fully optimize the formulation of media that, in many cases, have not been altered for several decades. This opportunity provides the potential to significantly increase the yield per liter of unconcentrated bulk antigen, which will further save on costs and animal facility capacity per year

Challenges Associated with a Transition to Animal-Free

One of the risks to companies when making a switch of the media used in a product is the costs and regulatory restrictions imposed by the governing agency that oversees the safety and efficacy of the products. These costs can vary from hundreds of thousands to tens of millions of dollars, depending on the extent of change and level of restrictions and testing imposed by the governing agencies (Constance, 2009). Because of these costs, there has been little incentive over the last half-century to change product lines that have reliably produced efficacious products at a reasonable expense. Unfortunately for those companies, the regulatory climate and

scientific reality has presented a situation where continuing to use a century-old manufacturing process is no longer feasible. Current clostridial vaccines are very inexpensive for the consumer to purchase. This restricts the manufacturer's return on investment to only a fraction of a cent per dose. In 2007, the cost to vaccinate a 100 head herd of cattle with a 7-way clostridial vaccine cost less than $25.00 (USDA-APHIS, 2008a).

Animal-free media have been available for many years, but have not been used in the manufacture of veterinary clostridial vaccines for a variety of reasons. Because of the limited revenue that clostridial vaccines provide for the companies, there is a limited budget or interest in altering the product lines if substantial costs are required. The most significant cost arises from regulatory requirements and optimization challenges. While it is a fact that in most cases, prepared vegetable-based powdered media are equal to or less costly than equivalent peptone or cooked-meat media, this is only a nominal portion of the costs that such a switch would demand. However, there is the need for research to identify an optimal medium composition that not only provides a good basal growth medium, but also has the appropriate conditions for antigen production – the expression of toxins and immunogenic cellular components. The ideal medium formulation will maximize not only the overall cell mass produced, but also the toxin produced per cell.

Unfortunately, very little is known about the expression requirements and timing for nearly all of the clostridial toxins. While it is known that a few of the toxins are expressed during sporulation, many are expressed during other growth

phases or are triggered by unidentified environmental stimuli. Additionally, there are no cost-effective and reliable *in vitro* tests for potency and efficacy, so nearly every clostridial antigen must be evaluated by expensive, restrictive and time consuming animal testing. These additional costs add to an already strained budget, and reduce the corporate incentive to voluntarily take on the challenges involved in switching to animal-free media. Along with the significant scientific challenges and financial costs described here, there are also the potentially significant challenges posed by regulatory bodies, such as additional media prion-testing requirements, reduced availability of prion-free stock herds, and expensive final product testing for prion contamination.

CHAPTER 5 – CONCLUSION

Discussion

Vaccination of cattle against the clostridials has been one of the most successful and cost effective means of ensuring a safe and reliable food supply in the history of modern agriculture. However, the challenges of the new and emerging threats posed by prions have set the stage for a change in how we think about the production of clostridial vaccines. While traditional meat- and animal-based media have held strong in veterinary vaccine production for most of the past century, the increasing availability and gradually falling cost of animal-free replacement media is slowly pushing the industry to a new standard.

The regulatory agencies that oversee the manufacturers worldwide have pushed hard on the human biologicals and pharmaceutical industries to remove all animal-based products and components from their manufacturing processes. It is only a matter of time before the same pressures are exerted on the veterinary industry. While it will be costly for vaccine manufacturers to change over to a new process, as well as to the end users who will have to pay for the additional costs through price increases, the cost of losing a vaccine product from the market because of changing regulations is much greater. Further, any additional costs and expenses incurred through these modifications will ultimately be passed on through the market

to the food producers, and ultimately onto the end consumer as higher prices at the grocery stores.

Recommendations for the Future

The veterinary vaccine industry is small relative to the overall biotechnology sector, but its impact on the health and safety of the food industry is tremendous. By moving quickly to a substantially animal-free manufacturing process for all clostridial antigens, there will be an even greater emphasis on food safety and the prevention of new outbreaks of frighteningly devastating disease.

Several veterinary vaccine manufacturers are already starting to experiment with a change to completely animal-free, plant-based media for production of clostridial vaccines. However, even those manufacturers have not yet succeeded in completely eliminating animal-based components from all of their clostridial product lines. The use of animal-based components will continue to grow as a significant threat to the human food supply, due to risks ranging from anaphylactic reactions to TSE transmissibility.

The regulatory agencies which provide oversight to the veterinary vaccine industry are under pressure to push new incentives and regulations on manufacturers so that complete incorporation of animal-free production can be rapidly and effectively introduced into all clostridial vaccine products. All of the vaccine manufacturers in the United States also export some of their products overseas,

meaning that the same products are licensed under multiple international regulatory agencies. The often contradictory and duplicative restrictions imposed by these agencies to altering vaccine production methods and formulations results in an industry-wide stagnation and unwillingness to change the status quo.

Using innovations developed from human pharmaceutical manufacturing processes in removing animal based components from their products, as well as the very recent developments of animal-free media for production of at least some of the clostridial vaccine strains, the risk of BSE-related infection from vaccination will rapidly be eliminated.

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