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## Investigation of Safe-Level Testing for Beta-lactam, Sulfonamide, and Tetracycline Residues in Commingled Bovine Milk

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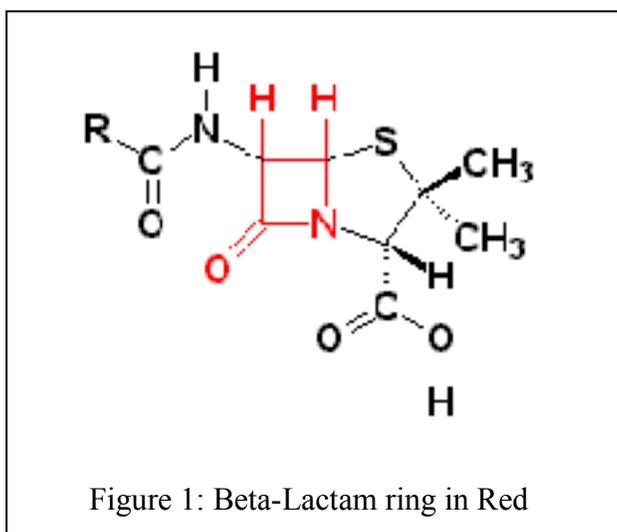
In the United States more than “a billion pounds of butter, 7 billion pounds of cheese, 1.5 billion pounds of yogurt, and one billion gallons of ice cream” (USDA, 2005) are produced annually. According to the USDA Food Pyramid, it is recommended that every person consume between two and three cups of milk products every day (USDA, 2005). With these large numbers in mind, one can easily see why a question is raised when it comes to what exactly is in dairy products and are these components harmful to humans? The greater question is, “What are the policies surrounding these antibiotic residues and their testing methods?”

In a 1988 study 71% of test samples from farms and the retail market tested positive for antibiotic residues; 63% of which were tetracyclines and sulfonamides in the United States (Milk Safety Branch, 1992). With such high percentages it can be seen that not only do the effects of these antibiotic residues need to be tested, but actual safe-levels of antibiotic residues for human ingestion must also be determined. Once safe-levels are determined, test methods that can be completed on a large or a small scale must be created in order to test for those levels. An international standard must be created to ensure justice to people worldwide. There must also be an education of farmers, veterinarians, and those in and around the dairy industry worldwide. This education must focus on the importance and hazards of safe protocol when dealing with milk and antibiotic residues on a regional, national, and international level.

Dairy cows commonly get pathogenic infections, which require antibiotic treatment. The most common and frequently occurring infection causes mastitis. Mastitis is an inflammation of the milk-producing glands causes great pain to the dairy cows. (Althaus, 2003) Some antibiotics are given to young calves to help them combat common bacterial diseases so they can grow to their full potential. These antibiotics include all of the antibiotics used in adult cows. (WHO, 2001) Depending on the individual cow’s metabolism of the antibiotic as well as the dose of

antibiotic being administered, some antibiotics can be excreted out of the cow in its milk. The majority of antibiotics found in milk include beta-lactams, sulfonamides, and tetracyclines. Penicillin, a beta-lactam antibiotic, is the most widely used antibiotic in the dairy industry.

The beta-lactam antibiotics include a series of drugs composed of one consistent structure, the beta-lactam ring (Fig. 1 in red) with side groups that can be modified to alter the biochemistry of the molecule.



Beta-lactam antibiotics act on the penicillin binding proteins. When bound, these antibiotics inactivate cell wall synthesis in a pathogen, thus killing the bacteria. (Jacoby, 2005) These are broad-spectrum drugs used to treat gram-positive and gram-negative bacterial infections. About 10% of all people given beta-lactam antibiotics have allergic reactions to them (Rossi, 2004).

The second group, the sulfonamides, is synthetic antibiotic that mainly targets streptococci. Many people have severe allergic reactions to sulfa drugs; medicines are clearly labeled if they contain a sulfa derivative. (Rossi, 2004) Sulfonamides are analogous to para-aminobenzoic acid and, through competitive binding, inhibit the synthesis of dihydropteroic acid, a necessary step in the folic acid metabolism of pathogens (Fig. 2). (Gaskins, 2002)

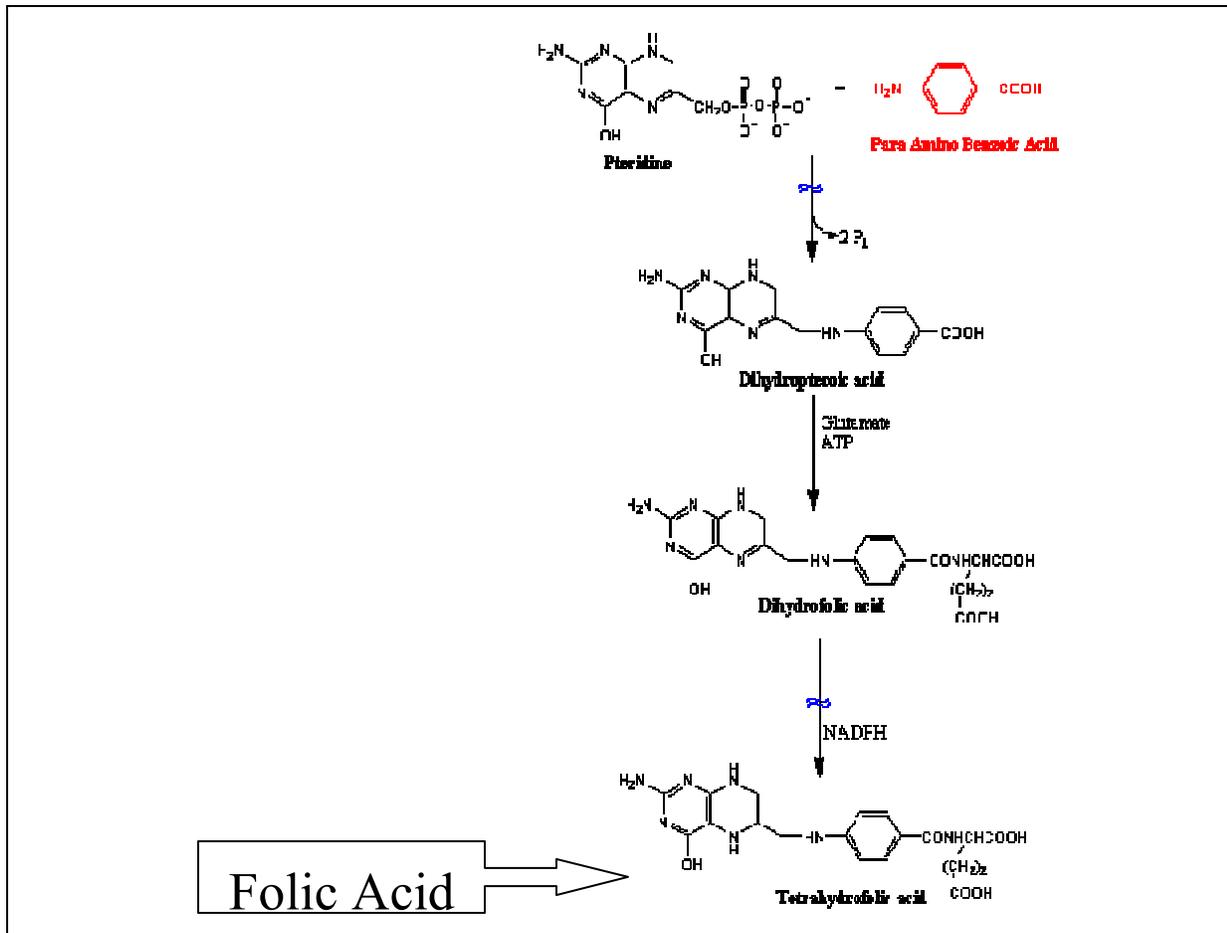
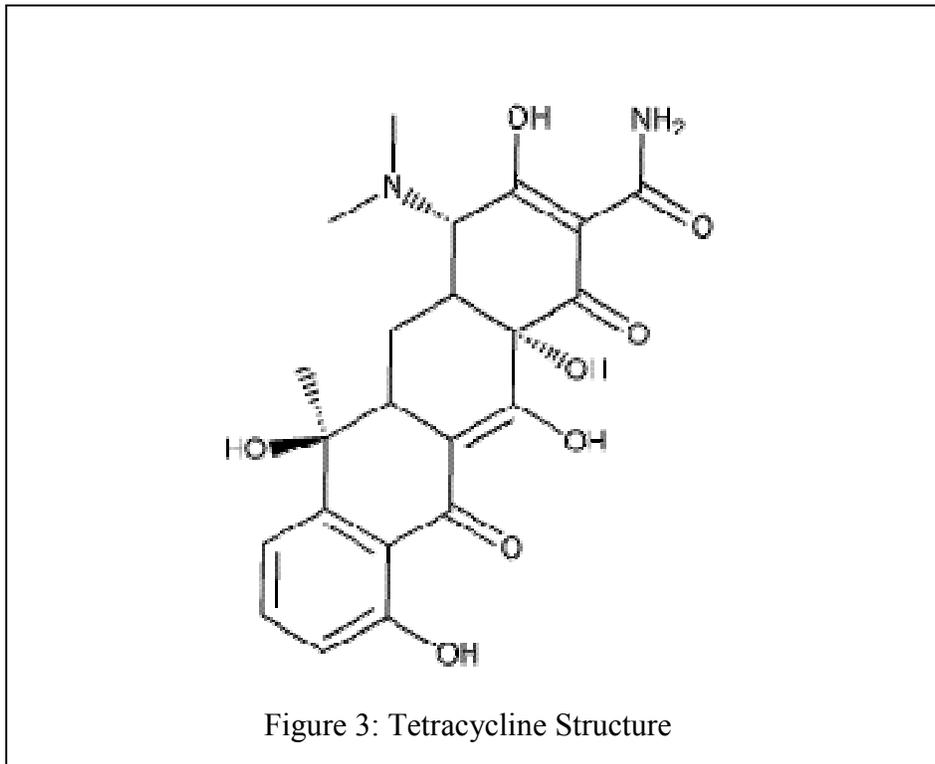


Figure 2: Folic Acid Cycle in Pathogen

If a pathogen is unable to produce necessary metabolites the pathogen will die.

Tetracyclines are also synthetic antibiotics used to target the streptomycetes bacteria. Tetracyclines also have a common ring structure (Fig. 3) and act by inhibiting protein synthesis in the pathogenic cell.



Particularly, this drug inhibits the transfer riboneucleic acid from binding with the ribosome in the bacterial cell; therefore transcription cannot occur, inhibiting the protein synthesis required for bacterial survival. (Chopra, 2001) Tetracyclines are very dangerous especially when taken by a pregnant mother, as they are teratogenic to the developing fetus. Also, tetracyclines are known to become more toxic after their expiration date. Thus, the use of “expired” tetracyclines as a common practice holds particular danger. When used improperly or over-used, all three of these above mentioned drug classes can be quite dangerous to the human population. Yet these are predominantly the drugs used most frequently in the treatment of dairy cow diseases.

Like in a human female, antibiotics can be excreted in breast milk. Thus, antibiotics and their metabolites get into the milk supply during milking. The antibiotics bind to proteins in blood serum and can be transferred from the blood into the milk. Of 37 common antibiotics Ziv and Sulman found that 75% of those antibiotics became bound in the serum at levels above 50% binding in the serum. (Ziv, 1972). When cows are on antibiotics their milk should not enter the milk supply. It is general practice to add milk from a cow that is receiving antibiotics only after

the recommended withholding time approved by the FDA. This duration would suggest that all antibiotic residue has left the body, however this is not always the case. Each cow is an individual and it can take longer, on a cow-to-cow basis, to leave the system. (Ziv, 1972). When antibiotic-contaminated milk is put into the bulk tank it contaminates the entire tank of milk. These accidents can occur due to the cow's individual metabolism to clear the drug, carelessness of a farmhand or greed to enter more milk into the bulk supply. Just looking at a vial of milk will give no information if it has been contaminated with antibiotics. Testing must be done to ensure no accidents have occurred. This is done at the dairy creamery. Milk from the bulk tank from the farm is transferred to the creamery where it is tested in a lab and then distributed to other factories to be bottled or made into other products. It is then shipped to retail stores where the consumer can purchase it for use. Different products made at the creameries include whole milk, skim milk, condensed skim milk, cream, and powdered milk. Milk containing antibiotic residues can get into any of these final products and be distributed for human consumption if not for rigorous safety standards and residue testing.

The potential hazards of ingesting antibiotic residues in contaminated milk include “allergic reactions, interference in the intestinal flora, and resistant populations of bacteria in the general population” (Althaus, 2003). Perhaps the most dangerous of these hazards is the potential for creating resistant populations of bacteria. The few bacteria that survive antibiotic treatment have a resistance to the antibiotic. This “super-bug” can reproduce more clones of resistant bacteria. Repeated ingestion of certain antibiotic residues from infected cows to humans can increase the likelihood of creating resistant bacterial forms. Population resistance refers to the resistance and tolerance acquired by a bacterial population to a given antibiotic. (WHO, 2001) The transfer of resistance occurs through an R-plasmid transfer. An R-plasmid is a small amount

of bacterial genomic DNA that codes for added proteins not normally found in the bacterial genome. Not only can this R-plasmid be transferred within species of bacterial, but intraspecifically as well-- this can include from a cow host to a human host. This transferable drug resistance causes great concern, especially because it can be transferred between species, because common bacterial infections that were once easily curable will soon become incurable and new drugs will have to be developed and tested. (Kruse, 1994) This will not only create a need for new drugs, but there will also be new bacterial species introduced into the population. Tolerance is gained through an excessive use of antibiotics as well as using too many broad-spectrum drugs to cure a bacterial or viral infection (Milk Safety Branch, 1992). Perhaps the biggest problem with resistance is that it is very hard to monitor. If a resistant pathogen is identified in a human host, it is not common to trace its origin to the milk supply. Many resistant pathogens could have arisen unwittingly from the milk supply. (WHO, 2001)

A second concern of antibiotic residues in milk is the chance of an allergic reaction. Allergies to antibiotics occur when the body's immune system attacks the antibiotic, which is often a haptenic metabolite of the antibiotic and some carrier tissue. (DeSwarte, 1984). Small levels of antibiotics can be very hazardous to susceptible humans causing acute to severe reactions. For those people with very low tolerance for allergens a small amount of residue could induce a severe allergic reaction, sometimes leading to death. These reactions occur because immunoglobulin IgE antibodies activate primarily the mast cells and basophiles, which are the effector cells in the immune system. The effector cells then cause an inappropriate response on the cells they activate, which in turn causes increased inflammation within the body. This inflammation can in turn produce various side effects at different locations in the body. (Hsieh, 2004) Although this may seem like a severe hazard, there are not many recorded allergic

reactions linked to antibiotic residues in milk. This is mainly because most physicians do not think to check all the way down to the antibiotic residue levels in milk when a person presents with an allergic reaction. Since these antibiotics are prominent in society and used to treat many common illnesses, the physicians generally find some other mode of reaction to blame. (Milk Safety Branch, 1992)

In addition to allergic reaction there is some indication in the science literature to suggest that antibiotics can induce cancer and other non-cancerous hazardous effects on the body. Drugs that were previously used as antibiotics such as Sulfamethazine and Nitrofurazone have been proven to be carcinogenic. Chloroamphenicol, found occasionally in milk, was shown to cause bone marrow disease. (Milk Safety Branch, 1992) However, if there is a prevalent link between severe health effects and antibiotic residues in milk, the FDA immediately recalls that antibiotic. As a result, antibiotics such as Sulfamethazine and Nitrofurazone are no longer used because of their carcinogenic characteristic. Hazardous effects occasionally cannot be determined during drug development because some of the side effects take many years to emerge. Unfortunately a few must suffer for the protection and safety of many.

There is an obvious need to determine the safe-level of residues of different antibiotic groups that find their way into the milk supply. These include finding the levels that are safe for human ingestion as well as the development of more rigorous, timely, and efficient testing methods. In the U.S. safe-levels are identified through the government agency Hazard Analysis Critical Control Point (HACCP). The HACCP is responsible for looking at different steps within general practice and making them safer. HACCP has a milk and dairy branch, working in conjunction with the FDA, to ensure proper steps will be taken. The FDA sets the safe-level by first identifying the lowest level of antibiotic that can be detected in a sample of milk. It then sets

this point as the most antibiotic allowed in the milk. (Sischo, 1996) The World Health Organization has left the determination of testing methods and safe-levels up to the responsibility of each individual country. However, the *Codex Alimentarius* was designed to set levels that are generally followed on an international scale. All countries do not currently adhere to these standards. (Hall, 2004) Here in the United States the FDA creates many national regulations, but leaves procedure up to the 55 milk-testing laboratories in the United States. Throughout Europe and all European Nation, the European Union determines the safe-levels. (WHO, 2001)

In order to test milk for safe-levels, different testing protocols and methods must be designed to produce accurate results at or near the safe-level standard. (Hall, 2003) There are tests that take a long time, such as the *Bacillus stearothermophilus* test and the use of high performance liquid chromatography (HPLC). The *Bacillus stearothermophilus* test uses this species of bacteria and a zone of inhibition to determine if antibiotics are present. The milk sample is placed on an agar plate inoculated with *B. stearothermophilus*. If a ring forms around the milk deposition bacterial growth has occurred, antibiotics present in the milk have created a zone of inhibition. Titration and standardizations using several control samples will allow one to estimate the amount of antibiotic residue in the sample. This time-consuming test is not designed for bulk testing nor does it allow for the quantification, in parts per billion, of antibiotic residues in the milk. It is not a good choice in an industry that needs to get accurate and precise results quickly. However, it is still used routinely in Canada, along with other testing methods. (Wehr, 2004) HPLC employs a series of chemical reactions that separate components found within the milk. As each component is eluted by size one can then determine its exact type and quantity. (Wehr, 2004) These are expensive machines; not all lab-testing facilities can afford them, even in a first world country. (Althaus, 2003)

Quicker, less expensive, tests are needed in an industry that handles so many billions of gallons of milk a year. The two main tests that have been approved internationally are the DelvoTest and the Charm test. The DelvoTest takes approximately two and a half hours to complete, while the Charm test takes less than ten minutes to run. (Wehr, 2004) The Charm test has been designed to detect all three of the major groups of antibiotics at very low levels, which is very effective in detection. The Association of Official Analytical Chemists has approved both of these aforementioned tests. (Milk Safety Branch, 1992)

The DelvoTest comes in two main forms: a laboratory-administered version to be used by a technician and an on-farm test that a farmer can conduct. The Delvo Test detects limits that are equal to the maximum amounts of residue limits for beta-lactams and sulfonamides, however it needs to be enhanced in order to detect tetracyclines at safe-level amounts. Agar, mixed with a pH indicator, is inoculated with *Bacillus sterothermophilus var. cardolactis*. A pill containing freeze-dried bacterial colonies and the milk are added to the agar and placed in an incubator at 46-48°C for two and a half hours. (Wehr, 2004) If the milk sample is negative for antibiotics, then the bacteria will grow and produce byproducts. The byproducts create an acidic pH causing the pH indicator to change from purple to yellow. This test result would be marked as “not found”, meaning it is negative for antibiotics or that the antibiotics are below the maximum safe-level. If antibiotics are present in the milk sample they would kill the bacteria; no color change would occur. Purple agar indicates the milk sample is positive for antibiotics at or above the established safe-level. (Althaus, 2003) This testing method is used on a regular basis in milk testing facilities in the United States, Canada, United Kingdom (European Union), and China. (Wehr, 2004; Hillerton, 1999; Chen, 1994)

The “on-farm” DelvoTest allows for the farmer to test his/her own cows. It is advantageous for a farmer to know beforehand if the cows are antibiotic positive; introducing tainted milk into the creamery would incur large financial penalties. Hillerton, et. al conducted an experiment in the United Kingdom on the on-farm DelvoTest. This study was to confirm the effectiveness and cause of repeated false positives. The results of this blind study of large-scale farms, independent producers, and test herds concluded that the main cause for false positives were improper test protocol. (Hillerton, 1999) To avoid this problem in the United States, regional governments allows farmers to send individual and bulk tank samples to have their milk tested by trained laboratory technicians. Many milk producers take advantage of this opportunity, thus avoiding the fees and harm associated with antibiotic residues and false positive tests. (Hillerton, 1999)

The DelvoTest is also useful in detecting antibiotic residues in goat ewes’ milk. (Wehr, 2004; Althaus, 2003) The test works in the same method as stated above. This is very helpful to the goat producers who often see very high rates of pathogenic disease. Sometimes this milk is too thick to run via other testing methods due to different component levels as opposed to cows’ milk. A test that is as accurate as the DelvoTest advantageous in the goat milk industry. Althaus et al determined that DelvoTest was useful and accurate in detecting antibiotic residues in goats’ milk. (Althaus, 2003)

The CharmSL (Safe Level) is used to detect beta-lactams, sulfonamides, and tetracyclines. The FDA approves the CharmSL test because the assay detects levels closest to the pre-determined safe-levels. In a 1992 Milk Safety Branch House committee hearing, the safe levels for the three groups of drugs were given as: “beta-lactams: 4.8ppb, tetracyclines: 200ppb, and sulfonamides: 10ppb” (in parts per billion). Salter, et al. completed the quantitative analysis and

the accuracy of the CharmSL test using beta-lactams, as well as non-beta-lactam drugs, in order to define the safe-levels. This study led to the federal certification of the CharmSL test. The countries mentioned previously that use the DelvoTest also employ the use of the CharmSL testing method. Many use the DelvoTest as a back up to the Charm test to ensure a check and balance system before the milk is sent out for further processing to avoid the report of a positive test to the government. (Salter , 2001)

Charm Sciences, Inc.®, maker of the Charm tests, provides testing material to the United States, Canada, and countries that follow the European Union and Codex Levels of testing. Charm Sciences® has also made different tests that account for the different safe-levels that each country has set. For the Charm Test there are individual assays for the three main antibiotic groups. (Charm Science® also produces a series of testing supplies for other antibiotic residues, not as prevalent and not required to be tested) Below are tables of the safe-levels for different antibiotics within the three groups as well as a comparison between the United States/Canada and EU/Codex standards.

Table 1a: U.S. Beta-Lactam Standards

Antibiotic	Charm SL Detection Level (ppb)	U.S. Regulation (ppb)
Amoxicillin	5.6	10
Ampicillin	8.5	10
Cephaprin	13.7	20
Ceftiofur	46	50
Penicillin G	3.6	5

Table 1b: EU/Codex Beta-Lactam Standards

Antibiotic	Charm SL Detection Level (ppb)	EU/Codex Regulation (ppb)
Amoxicillin	3	4
Ampicillin	3	4
Cefalexin	30	100
Cefazolin	12	50
Cefquinome	15	20
Ceftiofur	30	100
Cephapirin	6	60
Cloxacillin	25	30
Dicloxacillin	20	30
Penicillin G	2.4	4

Table 2: U.S. Sulfonamide Regulations

Antibiotic	Charm SL Detection Level (ppb)	U.S. Regulation (ppb)
Sulfadimethoxine	6.7	10
Sulfamethazine	6.2	10

Table 3a: U.S. Tetracycline Regulations

Antibiotic	ROSA Detection Level (ppb)	US Safe Level (ppb)
Chlortetracycline	150-300	300
Oxytetracycline	150-300	300
Tetracycline	30-90	400

Table 3b: EU/Codex Tetracycline Regulations

Antibiotic	Detection Level (ppb)	EU/Codex MRL (ppb)
Chlortetracycline	70-100	100
Oxytetracycline	70-100	100
Tetracycline	15-30	100

(Charm Sciences, Inc ®, 2007)

Although these tests are utilized in many countries, other countries do not test milk at all or their testing methods fall below the standards. Although the *Codex Alimentarius* is currently the accepted international standard, each country can decide for itself what levels and tests are acceptable. Since the World Health Organization has not developed a global policy surrounding the testing methods or actual safe-levels, there is an inequality in the world when it comes to the

quality of milk products. The FDA, as well as the European Union, approves the few tests mentioned above, yet there are still many countries that lack testing technology or requirements surrounding the testing of milk. Inadequate testing technology does not produce the same accurate results as the DelvoTest and Charm tests do. It is incumbent upon the global community to stress the importance of these tests, set a standard, and ensure that the best testing methods are made available for all nations. (WHO, 2001)

Global policy should also include education on many levels. Safe milk supply begins with the farmers and pass through the dairy and food industry. Veterinarians and government officials are the last safeguard. All these parties need a firm understanding of the health implications of tainted milk. Perhaps the best example of the lack of education among dairy farmers can be found in an experiment completed by Chitandi and Sternesjo in Njoro, Kenya. This study compared variation of the number of positive samples on small versus large-scale dairy farms. They found that both large and small farms tested positive for antibiotics in their milk. However, the researchers found that the small-scale farms, on the whole, produced worse results. To determine the reason for this high percentage of positive samples, a questionnaire was designed. Three main problems were identified: “(i) lack of understanding of risks related to antibiotic contamination in food, (ii) poor or no treatment records, and (iii) lack of a monitoring system as major risk contamination.” (Shitandi, 2004) Even though this experiment focused on farmers in Africa, there is reason to believe lack of education in many countries results in tainted milk. There are positive milk samples that occur in all countries. Educating farmers on the importance of accurate record keeping as well as adequate determination of the cows’ status, as well as alerting them to the hazards and risks associated with these residues would go a long way in counteracting the broad scope of safe milk production. Finally, the farmers must make sure

that their farmhands are getting appropriate training on how that particular farm operates. In the United States, many farmhands are immigrants and speak English as their second language. This language barrier could be creating problems in the system that can be relieved.

An excellent example of what education can do for a country's milk supply can be found in Barbados. Hall et al conducted surveillance on products coming from animal origin there from 1996 until 2003. This study found that 6% of the milk producers tested positive in the year 1996, however a steady decrease over the seven years of the experiment occurred. In 2003 it was found that only 1% of the milk producers tested positive for antibiotics. This is a tremendous decrease over a short period of time that can be attributed to personal visits from the researchers to the farmers to explain to them positive tests and the hazards of antibiotic residues. If this is something that can happen in a relatively small area that is a bit more underdeveloped than the United States and other larger nations, it is something that can be translated to the large scale and eventually come to fruition for many other countries. (Hall, 2004)

Thus this concurs with the study by Torrence that certain safeguards should be instated: a) balanced record keeping, b) monitoring of the farmers' practice, c) re-education of veterinarians, and d) development of stricter government standards. Record-keeping documents need to include which cows get treated, what type of medication, and what dosage they receive. (Torrence, 2003) A monitoring system would include random visits to different farms to assess the amount of hazard at that particular location. The visiting team would be comprised of independent evaluators avoiding conflict of interest inherent in all industries. (Torrence, 2003)

Veterinarians, not by lack of education or willfulness, are often part of the problem. In an area with many farms record keeping by veterinarians should include epidemiological analysis of pathogenic outbreaks and the antibiotic treatment regimes use to combat the outbreaks. This

would help map out how resistance is emerging in different pathogens regionally, possibly leading to new drug development. If veterinarians carefully track what is prescribed as medication they can also work in conjunction with the milk testing laboratories. For example, if there is a farm that continually repeats positive test results in milk, then there is obviously a need for that farmer to get a better understanding of the antibiotics and how they must be used correctly in the dairy industry. If a veterinarian is alerted to this farmer's problem, the veterinarian can educate the farmer and review the dosing instructions with the farmer to ensure there is an understanding of how to properly treat his herd of cows.

Finally there is a need for governments to be proactive about protecting the populace from antibiotic residues in milk. The World Health Organization, respected internationally, could proscribe the *Codex Alimentarius* as the international norm for the dairy industry. All countries would have to follow these standards and testing methods thus ensuring healthful milk supply across the globe.

The dairy farm industry is diverse, ranging from mega-producers to small family farms. The industry is dependent on the health of its herds and antibiotics, by necessity, are required to keep the milking cows healthy. A natural consequence of this required treatment is the production of antibiotic metabolites, which in high enough concentration are harmful to humans. Careful monitoring of antibiotic residues can govern the unnecessary introduction of these moieties into the milk supply. This will require the education of many parts of the enterprise and the creation of safe-level tests appropriate to keeping on top of the ever-changing market of the dairy industry. Thus, a global policy that includes education, monitoring, accurate and reliable tests, and global regulations must be enacted in order to ensure the justice and safety of people worldwide.

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