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Examensarbete / SLU, Institutionen för husdjurens utfodring och vård, **385**

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Abstract

One of the leading causes of food borne infections in the world is due to *Salmonella* by consuming poultry products including eggs and meat. According to US Food and Drug Administration (2009), 2 to 4 million cases of Salmonellosis in humans occur every year only in US. *Salmonella* causes wide range of diseases with enteric and typhoid fever, food poisoning, diarrhea and gastro-enteritis. Many serotypes of *Salmonella* do not have host specificity and cause disease in all kinds of animals and humans. *Salmonella* has capability to modify according to the changing environment and it can develop resistance against routine elimination practices of sanitation, chemical treatments and antibacterial drugs.

Newly established poultry industries in Pakistan are confronting various infectious diseases including *Salmonellosis* while in Sweden *Salmonella* prevalence in animal products consumed for humans is extremely low. Poultry feed is considered to be the main source of transfer of *Salmonella* into poultry flocks. Dust, cooling system and feed ingredients can be major sources of *Salmonella* contamination during the feed milling process. Feed ingredients and environment which harbors *Salmonella* can mix contamination in feed which results in the cross contamination from feed to the animals.

The aim of this master thesis was to isolate *Salmonella* and other *Enterobacteriaceae* during the milling process in the poultry feed industry of Pakistan. Furthermore, to introduce the techniques and prevention methods for the control of *Salmonella* which has been successfully practiced in Sweden and other developed countries.

During the study, four feed mills producing poultry feed and 20 poultry farms consuming the feed from same mill were visited. Samples were collected from different compartments to isolate and identify *Salmonella* and ENT in the poultry feed production line. These compartments included raw material storage (RM), dosing bin (DB), mixer (MX), pellet making (PM), cooler (CL) and finished feed packaging (FF) along with storage bin of poultry farms. The results showed a 65.8% average contamination rate in the feed which was considered as an alarming situation for the poultry feed industry of Pakistan.

There is dire need to develop a better understanding and assist poultry stakeholders to reduce *Salmonella* invasion in poultry industry in developing countries, including Pakistan. Routine bacteriological supervision of the feed production in factories needed to be monitored. Since *Salmonella* pathogenesis is complex, thermal process and pelleting may not be enough methods to eliminate the *Salmonella* completely from feed. Recontamination may be occurring during cooling or transportation. The future perspective of this study is to introduce the knowledge to keep the whole chain of poultry production free from *Salmonella*.

Sammanfattning

En av de främsta orsakerna till livsmedelsburna infektioner i världen beror på konsumtion av salmonellasmittade fjäderfäprodukter, inklusive ägg och kött. Enligt US Food and Drug Administration (2009), inträffar 2 till 4 miljoner fall av salmonella hos människa varje år bara i USA. Salmonella orsakar många sjukdomar med enterisk och tyfoid feber, matförgiftning, diarré och gastroenterit. Många serotyper av Salmonella har inte värdspecificitet och orsakar sjukdom i alla typer av djur och människor. Salmonella har förmåga att förändras beroende på förändringar i omgivningen och kan utveckla resistens mot rutinmässiga elimineringsmetoder av rengöring, kemiska behandlingar och antibakteriella läkemedel.

Nyetablerade fjäderfäindustrier i Pakistan möter olika infektionssjukdomar, inklusive Salmonella, medan det i Sverige är mycket låg risk för salmonella i animaliska livsmedel. Foder anses vara den viktigaste källan till överföring av salmonellasmitta till fjäderfäbesättningar. Damm, kylsystem och foderingredienser kan vara en stor källa för Salmonellakontaminering under fodertillverkningen. Foderingredienser och miljö som härbärgerar Salmonella kan orsaka kontaminering i foder, vilket resulterar i smittspridning från foder till djuren.

Syftet med detta examensarbete var att isolera Salmonella och andra *Enterobacteriaceae* under tillverkningsprocessen av fjäderfäfoder i Pakistan. Dessutom, önskar man introducera de tekniker och metoder för salmonellakontroll som med framgång har praktiserats i Sverige och andra utvecklade länder.

Under studien har man besökt fyra foderfabriker som tillverkar fjäderfäfoder och 20 fjäderfägårdar som köper foder från samma fabriker. Prover samlades in från olika avdelningar för att isolera och identifiera salmonella och enterobakterier i produktionslinjen för fjäderfäfoder. De undersökta avdelningarna var råvarulagring (RM), dosering (DB), mixer (MX), pelletstillverkning (PM), kylare (CL) och packningen av färdigt foder (FF) tillsammans med lagerutrymmet på fjäderfägårdarna. Resultaten visade en genomsnittlig kontaminering med 65,8% i fodret, vilket betraktas som en alarmerande situation för fjäderfäfoderindustrin i Pakistan.

Det är ett stort behov av att utveckla en bättre förståelse och att hjälpa fjäderfäintressenter att minska salmonellaspridningen inom fjäderfäindustrin i utvecklingsländerna, inklusive Pakistan. Rutinmässig bakteriologisk övervakning av foderproduktionen i fabrikerna behöver tillämpas. Eftersom Salmonellans patogenes är komplex, är kanske inte upphettningen under pelleteringen en tillräcklig metod för att helt eliminera salmonella i foder. Återkontaminering kan förekomma under kylning eller transport. Det framtida målet efter denna studie är att förmedla kunskap för att hålla hela kedjan inom fjäderfäproduktionen fri från salmonella.

Introduction

Background

Poultry is a viable and quick source of nutrition during food shortage for an increasing human population. However, newly established poultry industries in Pakistan are confronting various infectious diseases including Salmonellosis which is a vertically transmitted disease and results in decreased production and low weight gain with a flock mortality at up to 90%. The effusive use of antibiotics against this infectious disease is causing not only bacterial resistance but is also responsible for harmful residual effects in meat and eggs leading to health hazards for humans.

In developing countries, including Pakistan, poultry feed is still considered to be the main source of transfer of *Salmonella* into poultry flocks since there is no regulations or control for eradication of *Salmonella* in the poultry feed production chain. On the other hand, in Sweden and other European Union states, *Salmonella* prevalence in animal products for human consumption is extremely low which has been documented in many studies published by the Swedish Board of Agriculture and the National Veterinary Institute. In poultry, prevalence of *Salmonella* is less than 1% and findings in cutting plants and retail outlets are rare (SVA, 2005).

In Western countries measures are widely applied for reducing the risk of introduction of *Salmonella* on farms and chicken flocks (Immerseel et al., 2002). Fowl typhoid and Pullorum disease have been effectively reduced in United States by using the methods of testing and eradication. The progress in reduction of the incidence of *Salmonella* in poultry can be achieved by combined application of control measures. These measures includes the production of *Salmonella* free feed by testing programs, eliminations of pests & rodents, disinfection and effective cleaning of poultry houses and preventive treatments. Increased demand for poultry products, public health concerns, political pressures and poultry exports has made it priority for the poultry producers to prevent food born transmission of diseases to humans (Gast, 1997).

Human Losses by *Salmonella*

In 2006, the European Food Safety Authority's (EFSA) report illustrated that *S. enteric* contamination isolated from foodstuff had caused human health problems with more than 170,000 cases every year only in European Union. During another survey in United States in 2005, 45 thousand cases of non-typhoid *Salmonella* were reported with an estimation of 1.4 million infections and 600 deaths every year (Murray et al., 2009). According to US Food and Drug Administration (2009), 2 to 4 million cases of Salmonellosis in humans occur every year only in US.

Diseases

In humans, *Salmonella* causes a wide range of diseases with enteric and typhoid fever, food poisoning, diarrhea and gastro-enteritis (Archer & Young, 1988; Lax et al., 1995). In poultry, it causes a variety of acute and chronic diseases (Gast, 1997) including paratyphoid, pullorum, fowl typhoid, avian arizonosis, enterotoxigenic diarrhea, plague, shigellosis and many more (Hirsh et al., 2004). *Salmonella* can infect poultry flocks via feed, water, hatching eggs and through environmental factors including birds, insects, rodents and farm workers (Wray & Wray, 2002). Onset of the disease may occur between 6-8 hours with an infective dose of 15-20 cells which is dependent on strain and health of host. The symptoms of disease are acute with prolonged effects of abdominal cramps, fever and mild diarrhea. This organism penetrates into the small intestinal epithelium from lumen of the gut where it causes inflammation and produces enterotoxin (US Food and Drug Administration, 2009).

Antimicrobial Resistance

Bacterial resistance to antimicrobial drugs is an old ecological phenomenon (Krügel, 1997) which increases the cost, mortality and morbidity of the disease. Antimicrobial resistance has a major concern with medical and veterinary sciences in relation to affect public health with social and economical consequences (WHO, 1997). The first *Salmonella* resistance was reported in early 1960s in Germany (Bulling et al., 1973), while Sojka and Hudson (1976) confirmed high level of resistance in *S. typhimurium* isolated in Great Britain in 1976. Swedish researchers found low resistance level of *Salmonella* due to less use of antibiotic in animal feed (Björnerot et al., 1996). Davies (1996) found that most of the *Enterobacteriaceae* family including *Salmonella* is resistant to the drugs including Aminoglycosides, beta-lactams, trimethoprim and chloramphenicol. Cohan and Tauxe (1986) also found in their observations that *Salmonella* as a resistant bacteria can create complications while treating other infections with antibiotics. Today, the industrialized countries have agreed that the development of bacterial resistance must be prevented in order to control the spread of these bacterial pathogens to humans (Helmuth, 2000).

Aim of the study

The aim of this study was to isolate *Salmonella* and other *Enterobacteriaceae* during the milling process in poultry feed industry of Pakistan. The reduction of the contamination in poultry products is the main goal which can be achieved by implementing the Swedish technique for *Salmonella* control in the feed supply chain. Application of ‘‘Hazard Analysis Critical Control Point (HACCP)’’ principles would be an effective mechanism to achieve the maximum safety of eggs and meat, produced from the flocks, destined for human consumption. Furthermore, the introduction of the ‘‘methods of prevention’’ can reduce the exposure and spread of *Salmonella*. Consequently, it would also be economically beneficial for both feed millers and poultry farmers who are rearing broilers for human consumption.

Literature review

Salmonella

Taxonomy

Salmonella are mostly defined as medically important gram-negative rods, non-spore forming; 2-4 × 0.5 µm (Jordan & Pattison, 1996), motile by flagella and most heterogeneous bacteria. These microbes belong to the *Enterobacteriaceae* family with more than 40 genera and hundreds of species and subspecies. These can be found worldwide in soil, water, vegetation and normal intestinal flora of many animals, including humans. Common medically important, *Enterobacteriaceae* family includes species of *Salmonella, Proteus, Escherichia coli, Klebsiella, Shigella, Morganella, Enterobacter, Citrobacter, and Serratia* (Murray et al., 2009).

Salmonella in poultry

The process of colonization of *Salmonella* covers humans and animals including livestock, poultry, rodents, reptiles and birds (Hirsh *et al*., 2004). The first case of *Salmonella* infection in poultry was reported in 1899. Most common age for infection in poultry is under 2 weeks and rare over 4 weeks of birds. The whole flock can be affected with 100% morbidity and less than 20% mortality rate. All serotypes of *Salmonella* have almost similar clinical signs with depression, ruffled feathers, diarrhea, swollen eyelids and sudden death (Jordan & Pattison, 1996).

Host Specificity

Many serotypes of *Salmonella* including *S.typhimurium, S. enteriditis, and S. infantis* etc do not have host specificity and cause disease in all kinds of animals and humans. *S. typhi*, *S. paratyphi* and *S. choleraesuis* are highly adapted to humans and cause severe diseases (Murray *et al*., 2009). In poultry, *S. pullorum and S. gallinarium* commonly cause Pullorum disease and fowl typhoid. These infections can be ingested through feces, fluff, litter and water (Hirsh *et al*., 2004).

Salmonella viability in feed

Estimated survival time of *Salmonella* in poultry feed is more than 98 days (Juven et al., 1984). Nashed (1986) found that viability of *S. typhimurium* in feed, at room temperature, is 71 weeks and in litter, 78 weeks. Furthermore at 7^{o}C the organism may survive up to 79 weeks in feed and litter. More than 80^{o}C temperature is required for the elimination of *Salmonella* from feed during steam conditioning (Blankenship et al., 1984).

Dissemination of *Salmonella*

Via poultry products

One of the leading causes of food borne infections in the world is still due to *S. enteritidis* by consuming poultry products including eggs and meat (Rabsch *et al*., 2001, Murray et al., 2009). Food poisoning in human beings is closely related by the use of poultry products which are contaminated with *Salmonella* (Nashed, 1986). Internal contents of eggs can also be contaminated by *S. enteritidis* (Gast & Beard, 1992). In a study, Campbell *et al.* (1982) found that birds contaminated with *Salmonella* may spread contamination to healthy birds due to *Salmonella* present in the environment. In poultry production cycle, various factors are responsible for the introduction of *Salmonella* including humans, rodents, feed, broiler house and hatchery.

Patrick et al. (2004) investigated that poultry eggs without proper cooking methods were a major risk factor for the outbreaks of *S. enteritidis* during the 1980s in United States. Furthermore, they described that control efforts prevent *S. enteritidis* illness from the region during the year 1985 through 1990. The National *Salmonella* Surveillance System (CDC) was developed to collect data of outbreaks from all locations. They included information of city, county, state, location of food preparation and consumption. The results from 1985 to 1995 showed that incidence rate of *S. enteritidis* increased from 2.38 to 3.9 per 100,000 population while with a decline of 49% it came down to 1.98 per 100,000 in 1999. The reason for this decline in infection and outbreaks could not be proved. It was thought that the implementation of prevention and control measures played a major role during the 1990s. These control measures mainly dealt with safe handling methods including proper cooking of eggs, regulations regarding refrigeration, quality assurance programs, educational messages, on-farm testing and traceability.

Capability of modification

In the 1980s, Shackelford (1988) reviewed in his study that *Salmonella* has capability to modify according to the changing environment. Moreover, it can develop resistance against routine elimination practices of sanitation, chemical treatments and antibacterial drugs. Mostly the responsible factors for the introduction of organism into the poultry production chain are feed, hatchery, poultry house, rodents and man. The infection in one bird can spread organisms internally or externally to all birds in the house. The poultry transport system provides the path for organism to transfer from one broiler delivery to at least the feathers of the birds of another delivery. He suggested that researchers and equipment manufacturer should emphasize on improving microbiological quality of poultry carcass. Furthermore, modification of advance techniques like automatic evisceration of carcass, spray scalding, immersion chilling and freezing of carcass in plastic bags can be valuable methods to reduce the microbial load on carcass.

Factors responsible for transfer of *Salmonella* contamination

Animals can get infections when are fed with *Salmonella* contaminated feed. In the region where endemic infection is well controlled or absent, *Salmonella* contaminated feed is a major source for introducing *Salmonella* in animal food production (European Food Safety Authority, 2008).

Shirota et al. (2001) investigated that Enteritidis strain of *Salmonella* isolated from poultry farms belonged to the same phage type and was genetically related with the *S. enteritidis* obtained from feed. This study was conducted during 1993-1998 in eastern Japan to observe the genetic relationship of *Salmonella* serotypes which were contaminating eggs through poultry feed in poultry layer farms. Samples were collected from 16 commercial layer farms and 19 serovars were cultured. The results from pulsed-field gel electrophoresis patterns showed that *enteritidis* strain of *Salmonella* serotype obtained from egg content and feed samples were genetically identical and belonged to single phage type. The researchers concluded that *Salmonella* contamination isolated from these commercial layer farms was transferred from the feed.

Cox et al. (1983) conducted a study to analyze the status of *Enterobacteriaceae* (ENT) and *Salmonella* in commercial poultry feed including mashed and pelleted feed. In mashed and meal samples, the ENT percentage was present with a ratio of 100% and 92% while *Salmonella* was 58% and 92% respectively. On the other hand, in pelleted feed, the ratio of *Salmonella* was 0% while ENT was 60%. The ENT isolated in all samples was *Klebsiella pneumonia, Enterobacter agglomerans* and *Enterobacter cloacae*. *Salmonella* could not be detected in the pelleted feed. The presence of other ENT in the same feed suggested the researchers to assume that pelleting method could not completely eliminate *Salmonella* and other pathogens. On the basis of these results, the study concluded that commercial pelleting may not be able to destroy *salmonella* completely since their heat resistance is similar to other microbes of the ENT family.

Veldman et al. (1995) conducted a survey to evaluate the contamination rate of *Salmonella* and *Enterobacteriaceae* species in poultry feeds and feed components. The survey was based on the data collected from the samples during July 1990 and April 1991 from the Dutch feed industry in Netherland. Overall rate of contamination was 10% out of 360 samples (10gm each) collected. The results showed huge difference between contamination rates for mashed feed used for layer-breeders (21%) and pelleted feed (1.4%). The rate of contamination observed in feed components was 31% in fish meal (130 samples), 4% in meat and bone meal (83 samples), 2% in tapioca (58 samples) and 27% in maize grits (15 samples). They did not find any *S. enteritidis*. Twenty eight isolated serotypes of *Salmonella* were different from routinely detected serotypes from poultry flocks. They concluded that pelleting of feedstuffs is an efficient method to reduce the contamination rate in the feed.

Alshawabkeh (2006) conducted a study to investigate the occurrence of *Salmonella* in the poultry feed industry in Jordan. He collected 1546 samples from north, south and middle regions of the country and isolated 36 serotypes of *Salmonella* which were confirmed and identified by biochemical and serological tests. *S. enteritidis* (22.22%) was the most common identified serotype including *S. infentis* (13.88%), *S. typhimurium* (11.11%), *S. group B* (11.11%), *S. group C2* (8.33%), *S. gallinarium* (8.33%), *S. group A* (8.33%), *S. Arizona* (5.55%), *S. rough strain* (2.77%) and other *Salmonella* (8.33%). The incidence of *Salmonella* contamination was found to be highest in percentage (3.5%) compared with other feedstuff mainly corn (0.1%) and meat meal (2.1%). On the basis of results he concluded that presence of *Salmonella* serotypes in carcasses and feedstuff plays a major role in distribution of infection in poultry flocks. He suggested that additional research is required to reduce the burden of *salmonella* by controlling the pathogen in feed.

Major sources of *Salmonella* dissemination during milling process

The data from the studies of Jones and Richardson (2004) confirmed that dust and feed ingredients can be a major source of *Salmonella* contamination during the feed milling process. They collected samples from 3 feed mills which were individually producing 100,000-400,000 tons of feed every year. Five different locations were selected for sampling from each mill including ingredient receiving area, mixer, pelleting mill, cooler and out loading regions. A total of 886 samples were collected with a range of 68 samples from feed ingredients, 189 samples from dust and 629 samples from feed. The temperature was also recorded for each sample which was collected from the pelleting mill. The results showed significantly higher *Enterobacteriaceae* counts in those feed samples which were also positive for *Salmonella* as compared to the feed samples which were not contaminated with *Salmonella*. The data illustrated that maintaining high temperature during pellet making was not sufficient to eliminate *Salmonella* and the distribution of contamination was also uneven. However, they suggested that 85^{o}C temperature is required during pelleting to eliminate *Salmonella* completely. They concluded that contamination rate was related with management practices of mill. Furthermore, dust and feed ingredients remained the main sources of contamination.

Davies and Wray (1997) conducted a study in ten animal feed mills in the United Kingdom and found that cooling systems were providing excellent medium for the growth of *Salmonella* colonization for both pellet and mashed feed. The range of *Salmonella* isolation in all feed mills varied between 1.1% and 41.7% depending upon the facility. On the other hand, in cooler it was as high as 85.7%. More than 3000 samples were collected from spillage and dust from roof of coolers and beneath the lids of storage bin. Finished products, out loading gantries and ingredient intake pits of four mills were also found to be contaminated with *Salmonella* by wild bird droppings. A variety of *Salmonella* serotypes were isolated including *S. typhimurium* and *S. enteritidis*. This study concluded that there is no clear guidance for effective conditioning temperatures in relation with time management while 80^{o}C temperature for at least one minute is considered to be successful for eradication of all contaminations. Furthermore there is also risk of recontamination in coolers even if the method of effective heat

treatment has been adopted. The persistent contamination with a high level and a wide range of *Salmonella* serotypes in storage bins was isolated due to poor ingredient selection.

Davies and Wales (2010) conducted an investigation to compare *Salmonella* status in poultry feed mills and on-farm home mixers in United Kingdom. They collected 1698 samples from 4 feed mills and 334 samples from 4 home mixers were collected during one or more visits. Samples from dust, spillage and aggregated material were collected from all parts of production lines in the feed mills, including silos, intake pits, elevators, weighing vessels, mixers, milling plant, conditioners, pellet presses, coolers, finish bins and out loading gantries. In home mixers, samples were collected from aggregated faeces, dust, livestock area, litter and bedding from pens, spillage from layer house machinery, surface swabs from cages, pens and drinkers. Furthermore, samples were also collected from wildlife and domestic animals of both feed mills and home mixers. A number of *Salmonella* serotypes detected during the investigation were associated with contaminations by ingredients, especially protein sources for feed mills. In comparison, the home mixers, the risk for *Salmonella* contamination was associated with farm environment, including harvesting equipment and storage areas. *S. typhimurium* DT41 was isolated from the grain drying area on a farm which was generally associated with wild birds. The rodents and wild birds had greater chances to transfer *Salmonella* from surrounding environment to the farm as compared to the feed mill. The use of chemical treatment for elimination of endemic *Salmonella* serotypes did not work efficiently. The uses of animal feed stuffs were a constant risk factor for *Salmonella* contamination in home mixers. The home mixer units were providing the source of recycling of farm *Salmonella* contamination into the feed. In conclusion they analyzed that dust and spillages debris around milling equipment was a more sensitive sampling technique compared to previously use direct sampling and monitoring methods.

Salmonella control measures in Animal feed

Jones (2011) reviewed practical *Salmonella* control measures in animal feed. He suggested that control measures can be divided 3 broad categories. First of all, an attempt should be made to prevent the contamination from entering the facility. Secondly, a system can be developed to reduce conditions which enhance microbial growth within the processing facility. Thirdly, design the procedures which eliminate the pathogens. He described further that to assess the accurate contamination rate, it is essential to collect the samples aseptically from the feed materials which are supposed to enter the facility. Developing a system for continuous control of dust and oil or fat accumulations is a necessary task because it provides the excellent medium for the growth and spread of microbes within feed manufacturing facilities. Similarly, uncontaminated feed materials can be obtained by controlling rodents, wild birds, sanitation of transport vehicles and restricting personnel accesses to facilities and equipment. Hazard analysis approach can be used to discover and disrupt the locations involved in multiplication of *Salmonella*. He concluded that thermal process and pelleting may not be enough methods to eliminate the *Salmonella* completely from feed and recontamination may occur

during cooling or transportation. The goal of controlling the *Salmonella* can be achieved by the addition of chemical compounds including organic acids and formaldehyde.

Maciorowski et al. (2006) discussed the role of feed management practices to control food borne *Salmonella* species in animal feed and feed ingredients in a review study. They acknowledged that feed ingredients and environment which harbors *Salmonella* can mix contamination in feed which results in the cross contamination from feed to the animals. Antimicrobial compounds and management strategies are being developed to reduce the colonization and elimination of *Salmonella* from gastrointestinal tract of the animals. Feed additives including Prebiotics and Probiotics can be used to control the microbes in the intestinal tract while use of antibiotics may be prohibitive due to production cost factor. Concluding this review they suggested that use of HACCP approach to identify critical areas and to develop more efficient monitoring and sampling techniques for individual feed batches would minimize the impact of *Salmonella* in the future.

Himathongkham et al. (1996) studied the destruction of *Salmonella* in poultry feed by heating methods. They developed a relation with quantitative effects of time, temperature and moisture. Feed samples including feed mash, fresh pellets and pellets at loading were used in the experiments which were obtained from a feed mill in California. They used randomized factorial design for experiments to investigate the effects of certain factors (time, temperature and moisture) for *S. enteritidis*, *S. typhimurium* and *S. haardt*. The feed was processed with different levels of temperature (71.1^{o}C, 76.7^{o}C and 82.2^{o}C), time (0, 20, 40, 80 and 160 seconds) and moisture (5%, 10% and 15%). The results for rate of thermal destruction at 82.2^{o}C showed a linear relationship when logarithms of surviving *S*. enteritidis were plotted against logarithms of heating time which indicated that death rate of *Salmonella* decreased with standard heating time. Other temperatures showed similar linear relationships. On the basis of the results from these experiments, the researchers predicted that 93^{o}C heat treatment for 90 seconds with 15% moisture content is required for a 10,000-fold reduction of viable *Salmonella*.

Ashraf et al. (2005) studied effects of various Probiotics to control *Salmonella* infection and their performance on growth of broiler chicken in Pakistan. Eight groups of 160 day old broiler chicks were fed with non-pathogenic microorganisms, including *Lactobacillus acidophilus, Aspergillus oryzae* and *Streptococcus thermophilus*, daily for two weeks with dose rate of $\geq 10^{8}$ CFU/L in drinking water. These microbes were administered singly or in several combinations. At the age of three weeks 10 birds from each group were challenged with *S. gallinarium* by a dose rate of 1×10^{6}/L in drinking water. Researchers observed no mortality after the exposure of *Salmonella* infection which indicated the effective role of Probiotics in the control and prevention of *Salmonella*. Furthermore, there was high intake of feed and water which resulted in high weight gain in these groups.

Comparison of *Salmonella* control policies

Kangas et al. (2007) compared two *Salmonella* control policies in Finland, the Finish *Salmonella* Control Programme (FCSP) and the Zoonosis Directive 92/117/EC, to analyze *Salmonella* control cost and benefits in primary and secondary production for broilers. Furthermore, direct and indirect losses in humans due to *Salmonella* infections during the year 2000 were also included in this study. The Zoonosis Directive indicate measures for *S. typhimurium* and *S. enteritidis* in breeding flocks of poultry while FSCP handle all *Salmonella* serotypes in all levels of live animal production, including cutting plants. To develop the method of the study, the analysis of cost was divided into seven subcategories including official *Salmonella* control cost, additional control of primary and secondary production, market disturbances, feed control, additional control costs to society, public health losses and losses due to premature death in humans. Based on the calculations and cost assessment, the researchers concluded that FSCP control policy was more successful compared to EU Zoonosis Directive because prevented loss of one human life covers the control cost in broiler production chain.

Comparison of cultural methods for *Salmonella* isolation

Koyuncu and Häggblom (2009) conducted a study to compare and verify the reliability of various standard cultural methods which are used for the isolation of *Salmonella* in feed ingredients. The NMKL71 method (Nordic Committee on Food Analysis) was compared with international standard method (EN ISO 6579:2002) and Modified Semisolid Rappaport Vassiliadis method (MSRV). Feed materials including wheat grain, rapeseed meal, palm kernel meal, soybean meal, pellets of pig meal and scrapings from feed mill elevators were investigated. The *Salmonella* serotypes including *S. typhimurium*, *S. cubana* and *S. yoruba* were artificially added to each feed ingredient at four different levels. On the basis of results they concluded that accuracy, sensitivity and specificity for all feed materials and *Salmonella* serotypes was surprisingly equivalent and there was no difference in detection levels when comparing all three methods.

Reliable techniques for isolation of *Salmonella*

Frederick and Huda (2011) explained briefly in their review study about the importance to adopt reliable techniques for isolation and identification of *Salmonella* species in poultry feed and poultry house environments in order to reduce the spread of *Salmonella*. They found that the accurate reporting system makes it essential to develop the effective and reliable methods for detection of *Salmonella* pathogens. The most reliable techniques that can be used for isolation and identification of *Salmonella* species are conventional cultural methods and Polymerase Chain Reaction (PCR) based techniques. The PCR based techniques include Pulsed Field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic (RAPD) and Entobacterial Repetitive Intergenic Consensus PCR-fingerprinting (ERIC). They concluded that incidence of the infestation of *Salmonella* in poultry houses is more likely to be transmitted to the birds which increase the risk for the exposure of Salmonellosis to the humans. Furthermore, a better understanding of effective control measures will assist farmers and feed millers

to reduce *Salmonella* in poultry feed, rearing environment, slaughterhouses, poultry meat and egg products.

Salmonella isolation from animals and feed stuff in Sweden

Sweden has adopted more strict regulations than other countries in order to be able to reduce the surveillance of *Salmonella* in animals. The reports of *Salmonella* isolations from environment, feed stuffs and animals are send to authorities, including the Swedish Board of Agriculture (SJV), the State Epizootiologist and the official country veterinary officer since 1961. The primary isolates are made, confirmed and serotyped by National Veterinary Institute (SVA). These two authorities have been involved in publishing a series of four year reports based on statistical records about *Salmonella* in animals and in feed stuff since 1949 (Thal et al., 1957; Rutquist & Thal, 1958; Karlsson et al., 1963; Hurvell et al., 1969; Gunnarsson et al., 1974; Sandstedt et al., 1980; Mårtensson et al., 1984; Boqvist et al., 2003).

Mårtensson et al. (1984) published a report about *Salmonella* occurrence in feed stuffs and animals in Sweden based on records during 1978-1982. During this period, 1266 outbreaks of *Salmonella* were reported in domestic and wild animals. Previous reports documented 835 outbreaks during 1963-1967, 1746 outbreaks during 1968-1972 and 1106 outbreaks during 1973-1977. This large variation of the figures was due to outbreaks in wild animals which were brought for bacteriological examination. In poultry, the total number of outbreaks was found to be 220 during 1978-1982, which were almost the double, compared to the number found in the last report of 1973-1977.

Out of 220 outbreaks, 190 (87%) were reported only in chicken and hens which reflected the dominance of poultry production in Sweden during that period. In 35 isolated serotypes, *S. typhimurium* was most the commonly isolated serotype (17%). Other common isolated serotypes included were *S. livingstone* (13%), *S. liverpool* (9%), *S. agona* (7%), *S. infantis* (6%), *S. havan* (5%) and *S. mbandaka* (5%). The absence of *S. gallinarium* and *S. pullorum* from outbreaks proved the effectiveness of the *Salmonella* eradication program.

In feed stuffs, dust and scrapings, 524 strains belonging to 47 serotypes of *Salmonella* were isolated from a number of feed factories. The raw material contained 172 strains including 153 strains from animal origin and 19 strains from vegetable origin. Domestic meat meal was the most frequent contaminated material containing 147 strains. In poultry feed, 121 strains belonging to 10 serotypes were isolated from the finished product which was connected with the contamination of the raw materials.

During the investigation, it was found that *S. mbandaka* and *S. liverpool* was deposited on the bottom and coating of walls of a raw material silo in one compound feed producing factory. This stationary source was continuously contaminating the processing line.

The rise of outbreaks during 1981-82 was related with the distribution of contaminated poultry feed from feed factories. The condensation in cooling system of milling line made the coating of pelleted feed wet and provided excellent substrate for the growth of *Salmonella* which then easily passed into cooling system with dust material.

To reduce the chances of contamination through raw material, the SJV is monitoring all the imports of feed stuffs to the Sweden. With the help of sampling methods they make it sure that contaminated feed stuff will not be allowed to enter the country.

Boqvist et al. (2003) conducted a study to evaluate the status of *Salmonella* isolates from animals and feed production in Sweden during 1993-1997. The data was collected from SVA and SJV reports. It was based on the isolates obtained from cattle farms, pig farms, kennels, water in reptile terrariums, autopsies and sanitary slaughter. They also considered lymph nodes collected at the surveillance at the slaughter houses, follow up sampling at the farms and feed production including feed stuffs and feed mills. During the period, 87 serotypes were detected from 555 isolates, including 30 new serotypes which were isolated for the first time in Sweden. *S. typhimurium* (n=91) and *S. dublin* (n=82) were the most commonly isolated serotypes.

In poultry, the number of *Salmonella* isolates obtained from layers was 56, compared to 21, in broilers. The reason of this high number in layers was due to earlier implementation of *Salmonella* control program only in broiler production. The researchers found that the number of isolates has decreased during this period compared to previous years.

In feed production, the samples were collected from critical control points of the milling line and included dust samples and scrapings. From poultry feed milling lines, a minimum of 5 samples were collected weekly while 2 samples were collected from animal production lines. A total of 464 samples remained positive for *Salmonella* from critical control points including *S. livingstone* (n=62), *S. senftenberg* (n=37), *S. cubana* (n=35) and *S. mbandaka* (n=30). Most common serotypes, *S. typhimurium*, *S. enteritidis* and *S. Dublin*, were rarely isolated. This reduction of *Salmonella* contamination could be achieved by using the *Salmonella* free raw materials for feed production. The imported raw materials, which remained *Salmonella* positive, were soybean meal, maize and rapeseed products. The raw materials of vegetable origin had 194 *Salmonella* isolates. Furthermore, the contaminated raw materials were going through a decontamination process before mixing in production line.

By comparing the findings of this study with previous five years report, they concluded that the contamination rate remained similar in the feed sector. They suggested that control on *Salmonella* has been successfully achieved by the application of principles of HACCP throughout the production chain. The monitoring of the production line has proved to be an effective method to prevent contaminations from the feed.

It is common that during monitoring or any other sample collection, if *Salmonella* is isolated, an investigation is started without any delay and the farm is placed under restrictions. A retrospective longitudinal study was conducted on 112 *Salmonella* infected Swedish cattle farms. These farms were subjected to *Salmonella* restrictions between 1993 and 2002. Researchers wanted to investigate, how the potential risk factors were associated with the length of restriction period based on *Salmonella* infection. During the study, they found that EU accession of testing two negative herd tests had significant association with lower hazard, for release of *Salmonella* control restrictions (Boqvist & Vågsholm, 2005).

Salmonella Isolation in Pakistan poultry feed industry

Feed milling history

Pakistan is basically an agriculture country with a human population at around 180M. History of poultry feed manufacturing was developed in 1962, with a joint venture of PIA (Pakistan International Air Lines) and a multinational company named Lever Brothers. Till now, there are dozens of groups and companies who became successful while others failed in this business. Overall, poultry production became a significant enterprise and the backbone in the agriculture economy of Pakistan in later years (Rameez, 2009). In 2006, Rasool and Athar found in their survey that out of 120 poultry feed mills, 25 mills were equipped with automation and pelleting. The total Poultry feed production capacity was 5470 thousand tons while production during the year 2006 was 3975 thousand tons. Production is increasing every year.

Feed Milling Process in Pakistan

Most of the ingredients of feed pass through 6 general compartments during the milling process as described in figure 1. These compartments are comprised of raw material storage (RM), dosing bin (DB), mixer (MX), pellet making (PM), cooler (CL) and finished feed packaging (FF). The raw material is transported to the feed mill by the supplier which is processed directly or stored in silos. After the milling process, feed is commonly transported to the poultry farms in 50kg packing bags (Anonymous, 2011).

There are no proper rules or legislations, regulating sampling for testing any bacterial contamination from raw material before it is supplied to the feed mill. Even supplier has no awareness of handling the ingredients in accordance with relevant codes of practice or guides. Feed mills only check the fungal status if needed (Anonymous, 2011).

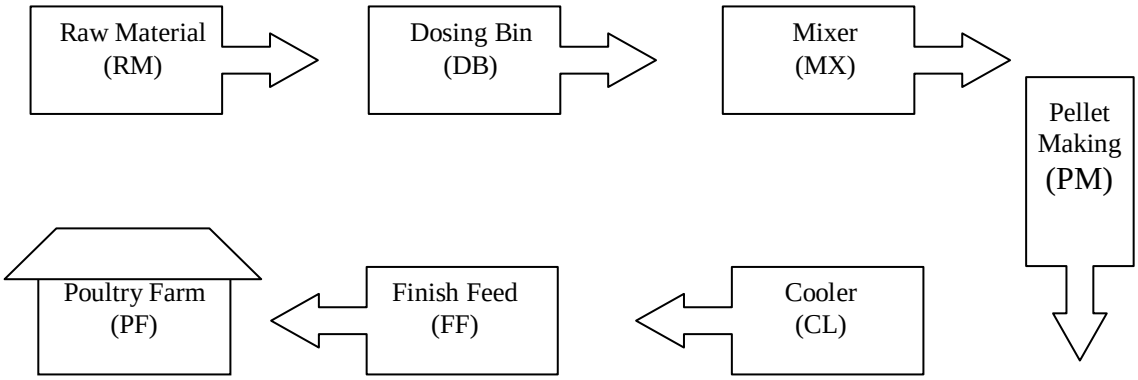

Figure 1. Scheme of feed processing in different compartments of Poultry feed mill.

Materials & methods

Sampling plan

Four poultry feed mills in central Punjab, Pakistan were selected for sampling. The feed mills were further categorized in alphabetical order as Mill A, Mill B, Mill C and Mill D. A total of 120 samples were collected during the spring 2011. Five compartments were selected for sampling, including Raw Material storage (RM), Dosing Bin (DB), Mixer (MX), Cooling (CL), Finished Feed (FF). The Pellet Making (PM) compartment was excluded because feed was processed at more than 80^{o}C during pellet making, a temperature when almost all microbes are killed. Five samples were collected from each compartment which included feed, dust and scrapings from walls and lids. Five poultry farms were followed for each feed mill and samples were collected from their storage bins. The farms were categorized with numbers as Farm 1, Farm 2, Farm 3, Farm 4 and Farm 5. One sample was collected from five different places including feed and dust of the storage bin of each farm. Each sample number from every compartment was categorized alphabetically as sample a, b, c, d and e.

A total number of 120 samples were collected from feed mills, including 20 samples from the poultry farms. Thus, from each mill, 30 samples were collected which included 25 samples from 5 compartments of the mill and 5 samples from five different farms which were supplied feed from the same mill. The weight of each sample was approximately 25-50 gm which was collected in sterile plastic glove and further packed in sterilized zip bags for proper labeling. The samples were transported at the same day under sterile conditions to the University Diagnostic Laboratory (UDL) at the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Samples were then stored at 4^{o}C for further processing. Each sample was investigated for the occurrence of *Salmonella*/*Enterobacteriaceae*.

Processing of samples

Enrichment of bacteria present in feed samples

The samples were inoculated in the Tryptic Soya Broth (TSB) for the enrichment and detection of the bacteria. The TSB (manufactured by LAB 4) (225ml) was prepared by adding 6.75gm of TSB in 225ml of distilled water, in 250ml glass flasks. The flasks were gently swirled and covered with aluminum foil. After wrapping the mouth and properly labeling, the flasks were autoclaved at 121^{o}C for 15min. The flasks were removed from autoclave and were kept at room temperature. The feed samples were removed from 4^{o}C to normalize their temperature. From each feed sample, 25gm was weighed and transferred into sterilized zip bags. The properly labeled zip bags containing 25gm of each feed sample and flasks containing 225ml Tryptic Soya Broth were transferred to the biosafety cabinet. These flasks were then incubated at 37^{o}C for 24 hours in the incubator.

Primary Culture on Selective Media for *Enterobacteriaceae*

After 24 hours incubation, the flasks were removed from the incubator and further cultured on the selective media for isolation of *Enterobacteriaceae* family members. The MacConkey agar plates and enriched incubated bacterial culture broth flasks were transferred to the biosafety cabinet. A loop full of that bacterial broth was streaked onto MacConkey Agar plates. Properly labeled plates were incubated at 37^{o}C for 24 hours in the incubator.

For the preparation of selective media plates, 51.5 gm of MacConkey Agar (manufactured by Scharlau 01-118) was weighed and dissolved in 1000ml of distilled water in glass flask. The flask was gently swirled and covered with aluminum foil. The flask mouth was wrapped and autoclaved at 121^{o}C for 15min. After autoclaving, the flask was removed from autoclave and kept at room temperature so that its temperature was decreased. As temperature decreased, the MacConkey media was poured in disposable Petri plates. Plates were allowed to solidify and were incubated for sterility in the incubator at 37^{o}C overnight.

Sub-Culturing on Selective Media for *Enterobacteriaceae*

After 24 hours incubation, the plates were removed from the incubator and further subcultured on the MacConkey Agar to get purified yellow and pink colonies separately. Single pink (lactose fermenting) and yellow (lactose fermenting) colonies were separately streaked on the MacConkey plates. Properly labeled plates were incubated at 37^{o}C for 24 hours in the incubator.

Biochemical tests

After 24 hours incubation, the plates were removed from the incubator and the Indole test was performed for further identification of bacteria. Single purified colonies from plates were inoculated into tubes and properly labeled tubes were incubated at 37^{o}C for 48-96 hours in the incubator. After incubation, the tubes were removed and Kovac's Reagent was added in tubes. The appearance of red ring showed positive while pink ring showed negative results. For

Salmonella/Enterobacter sp., pink ring was formed and this Indole test was found to be negative for *salmonella* (Negative Indole test shows presence of *Salmonella/Enterobacter* sp.).

To prepare media for Indole Test, 20gm of peptone (bacteriological peptone from meat manufactured by BIO BASIC INC.), 5 gm of NACL (MERCK), were weighed and dissolved in 1000ml of distilled water. A pH of 7.4 was maintained for this medium and 10ml solution was poured into 15ml test tubes. The tubes were properly labeled and autoclaved at 121^{o}C for 15min. After autoclaving, Indole Test Tubes and Sub-cultured MacConkey plates were transferred into a biosafety cabinet.

For the preparation of Kovac's reagent, 10gm of 4-dimethyl aminobenzaldehyde was weighed and added in 50ml of concentrated HCL; 0.5ml of this reagent was added in tubes.

Inoculation on *Salmonella Shigella* Agar and Urease test

For confirmation of *Salmonella*, single purified colony of non-lactose fermenting bacteria from MacConkey plates was streaked on SS agar plates and also inoculated into urea broth tubes. Properly labeled plates and tubes were incubated at 37^{o}C for 24 hours in the incubator. After incubation, black centered colonies appeared on SS agar, showing the *Salmonella* colonies. In urea broth tubes, the Positive reaction resulted in pink color of broth while *Salmonella* occurrence resulted in no color change (yellow color) of broth.

To prepare SS Agar plates, 60 gm of *Salmonella Shigella* Agar (SS Agar manufactured by LAB 52) was weighed and dissolved in 1000ml of distilled water in glass flask. The flask was gently swirled and covered with aluminum foil. The mouth of the flask was wrapped and autoclaved at 121^{o}C for 15min. After autoclaving, the flask was removed from autoclave and kept at room temperature so that its temperature decreased. As temperature decreased the SS media was poured in disposable Petri plates. Plates were allowed to solidify and then incubated for sterility in the incubator at 37^{o}C overnight.

For Urea Broth preparation, 0.9gm of Urea broth (Urea Broth Base manufactured by LAB 131) was weighed and dissolved in 95ml of distilled water. Properly labeled flask was autoclaved at 121^{o}C for 15min. After autoclaving the flask was removed from autoclave and kept in room temperature so that its temperature was decreased. As temperature decreased, 5ml of Urea solution was aseptically added in flask and from this flask, 10ml of urea broth was poured in 15ml test tubes aseptically. The tubes were incubated for sterility in the incubator at 37^{o}C overnight. After incubation, SS agar plates and Urea Broth tubes were transferred into a biosafety cabinet.

Triple Sugar Iron agar and Motility Test

The Motility Test and Triple sugar Iron Agar (TSI Agar) Test was also performed for further confirmation of *Salmonella*. For Motility Test, a loop full of broth inoculated with bacteria was taken on a cover slip. The cover slip was inverted on a slide with a little support to see the motility of *Salmonella* under the microscope at 40X. *Salmonella* species give positive results for motility.

The preparation of TSI Agar plates was performed by dissolving 65gm TSI Agar (manufactured by OXOID, CM0277) in 1000ml of distilled water in a glass flask. The flask was gently swirled and covered with aluminum foil. After wrapping the flask mouth properly, labeled flask was autoclaved at 121^{o}C for 15min. The flask was removed from autoclave and kept in room temperature so that its temperature was decreased. As temperature decreased, the TSI Agar media was poured in disposable Petri plates. Plates were allowed to solidify and then incubated for sterility in the incubator at 37^{o}C overnight. After incubation, TSI Agar plates were transferred in biosafety cabinet. Single purified black centered colony from SS Agar plates was streaked on TSI Agar plates. Properly labeled plates were incubated at 37^{o}C for 24 hours in the incubator.

Remel RapID™ One Kit Fluid Method System for *Salmonella* Confirmation

For identification of *salmonella*, TSI Agar positive samples were inoculated into the Remel RapID™ One kit. The Rapid system, manufactured by Remel, is capable of identifying a variety of *Enterobacteriaceae* microbes (Evangelista et al. 2002).

Briefly, 2-3 *Salmonella* suspected colonies from agar plates were suspended into the Remel RapID™ inoculation fluid (2ml) to achieve a visual turbidity. Suspension was mixed thoroughly, vortexed and used within 15 minutes of preparation. The lid of the panel, over the inoculation port, was peeled off by pulling the tab marked “peel to inoculate” up and towards the left.

The entire content of the inoculation fluid tube was gently transferred into the upper right-hand corner of the panel by using a pipette. The inoculated port of the panel was re-sealed by pressing the peel-back tab back in place. After adding the test suspension and while keeping the panned, on a level surface, the panel was tilted back away from the reaction cavities at a 45 degree angle approximately. While titled back, the panel was gently rocked from side to side to evenly distribute the inoculums along the rear baffles while maintaining a level of horizontal position. The panel was then slowly tilted forward towards the reaction cavities. This evacuated all of the inoculums from the rear portion of the panel. The panel was returned to a leveled position. It was necessary to gently tap the panel on the bench top, to remove any air trapped in the cavities.

Incubation of Remel RapIDTM One Panels

Inoculated panels were Incubated at 35-37^{O}C in a non-CO_2 incubator for 4 hours. For ease of handling, panels were incubated in the chipboard incubation trays provided with the kit.

Scoring of Remel RapIDTM One panels

RapID^{TM} One Panels contain 18 reaction cavities that provide 19 test scores. Test cavity 18 is bifunctional, containing two separate tests in the same cavity. Bifunctional tests are first scored before the addition of reagent providing the first result, and then the same cavity is scored again after the addition of reagent to provide the second test result. Test cavities, 15 through 17, require Remel RapID ^{TM} One reagent and are designated with a box drawn around them. Bifunctional test 18, which used Remel RapID ^{TM} One Spot Indole Reagent, was designated with a box drawn around the reagent requiring test. While firmly holding the Remel RapID ^{TM} One Panel on the bench top, the labeled lid was peeled off over the reaction cavities by peeling the lower right hand tab up and to the left. Two drops of Remel RapID ^{TM} One Reagent were added to cavities, 15 (PRO) through 17 (PYR). By Reading and scoring cavities, 1 through 18, from left to right, using the interpretation guide, test scores were recorded in the appropriate boxes on the report form using the test code above the box for the bifunctional test. Two drops of Remel RapID ^{TM} One Spot Indole reagent was added to cavity 18 and was allowed, at least 10 seconds but no more than 2 minutes, for color development. Test cavity 18 (IND) was read and the scores were recorded in the appropriate box of the report form. The species of bacteria (*Salmonella*/*Enterobacteriaceae*) were identified by Remel RapID™ Software Programme.

Results

Details of the *Enterobacteriaceae* (ENT) and *Salmonella* serotypes found in processing compartments of four feed mills with poultry farms are described in Table 1 while number of positive samples with their percentage is summarized in Table 2. The method of presenting the results was adopted from Davies & Wray (1997) and Davies & Wales (2010).

Mill A was visited during April, 2011 and 30 samples were cultured from five processing compartments along with 5 samples from storage bins at five different poultry farms. *Salmonella* was not isolated from the feed mill compartments but other ENT could be detected. *Klebsiella pneumonia* was isolated from the mixer while *Klebsiella oxytoca* was isolated from the cooler. In total, 15 samples out of 30 were positive for ENT with a ratio found to be 50%. *Salmonella* species were isolated from samples collected from the storage bins at Poultry Farm 1 & 2 while Poultry Farm 3, 4 and 5 were contaminated with *Shigella* species.

Feed mill B was visited during April, 2011 and 30 samples were collected and cultured. *S. choleraesuis* was isolated from the dosing bin, the cooler and all five poultry farms. Furthermore, *Klebsiella oxytoca* was isolated from the raw material while *Shigella sp*. was detected from the finished feed. The overall ratio of contamination was 85%. All samples from Poultry Farm 1, 2, 3, 4, and 5 were found to be positive for *S. choleraesuis*.

Mill C was visited during May, 2011 and a variety of ENT and *Salmonella* was isolated from 30 samples. *Enterobacter aerogenosa* was isolated from the raw material, *Klebsiella oxytoca* from the dosing bin, *S. gallinarium* from the mixer, *Klebsiella pneumonia* from the cooler and *Shigella sp*. from the finished feed. The overall ratio of contamination was found to be 93.3%. *S. gallinarium* was also isolated from Poultry Farm 2 and 4.

Mill D was visited during May, 2011 and 12 samples were positive out of 30 cultured samples, thus giving a 40% contamination ratio. *Klebsiella pneumonia* was isolated from the raw material and *E. coli* from the dosing bin. Samples from the mixer, cooler and the finished feed were found to be free from any contamination. Poultry Farm 2 and 3 was found to be positive for *Salmonella* but species could not be detected.

Table1. Enterobacteriaceae and *Salmonella* serotypes found in processing compartments of four feed mills and storage bins of poultry farms.

	Mill			
Location	A	B	C	D
RM	*0*	*Klb. Oxytoca*	*Etb. aerogenosa*	*Klb. pneumonia*
DB	*0*	*S. choleraesuis*	*Klb. Oxytoca*	*E. coli*
MX	*Klb. Pneumonia*	*0*	*S. gallinarium*	*0*
CL	*Klb. Oxytoca*	*S. choleraesuis*	*Klb. pneumonia*	*0*
FF	*0*	*Shigella sp.*	*Shigella sp.*	*0*
PF	*Salmonella sp. Shigella sp.*	*S. choleraesuis*	*S. gallinarium*	*Salmonella sp.*

RM, Raw material; DB, Dosing bin; MX, Mixer; CL, Cooler; FF, Finished feed; PF, Poultry Farms.

Klb., Klebsiella; Etb., Enterobacter; S, Salmonella; sp., species.

Table2. *Salmonella* and ENT isolation from samples of Poultry feed Mill compartments and farms.

	No. of samples positive for *Salmonella* and ENT/number of samples tested (%)					Total
		Mill				
Location	No. of samples	A	B	C	D	
RM	20	0	5	5	5	15/20 (75%)
DB	20	0	5	5	5	15/20 (75%)
MX	20	5	0	5	0	10/20 (50%)
PM	0	0	0	0	0	0
CL	20	5	5	5	0	15/20 (75%)
FF	20	0	5	5	0	10/20 (50%)
PF	20	5	5	2	2	14/20 (70%)
Total	120	15/30 (50%)	25/30 (83.3%)	27/30 (93.3%)	12/30 (40%)	79/120 (65.8%)

RM, Raw material; DB, Dosing bin; MX, Mixer; PM, Pellet Making; CL, Cooler; FF, Finished feed; PF, Poultry Farms.

Discussion

Previously, *Salmonella* and ENT contaminations have never been identified in feed industry of Pakistan. This investigation was conducted for the first time in order to isolate and evaluate the status of *Salmonella* in the poultry feed industry. The average contamination ratio detected was 65.8% (Table 2) including all four poultry feed mills and poultry farms. This is a quite high percentage as compared to the developed countries. It may be considered as an alarming situation for the health of the poultry and furthermore, for public health. Moreover, there is no legislation introduced to monitor the feed mills and the poultry production chain. In comparison, the legislation for the control of *Salmonella* in animals and feed stuffs has been introduced for decades in Finland. The annual prevalence in commercial broiler flocks was 0.5-2.9% during 1990-1994 (Maijala *et al.*, 2005).

In total, seven different species of ENT and *Salmonella* could be detected (Table 1) from 120 samples which includes *S. choleraesuis, S. gallinarium, Shigella sp., E. coli, Klebsiella pneumonia, Klebsiella oxytoca* and *Enterobacter aerogenosa*. The presence of high ratio of ENT in feed samples indicated the severity of the problem. Veldman et al. (1995) used *Enterobacteriaceae* count as useful markers to detect the *Salmonella* contamination level.

In all feed mills, 75% of the samples were found to be contaminated from raw material. Häggblom (1994) observed that most of the *Salmonella* positive samples were derived from raw materials in the feed mills. Davis and Wray (1997) discussed various factors which might be responsible for a high contamination level in raw material ingredients. Most commonly, the management of the mill was responsible for the purchase and selection of the poor ingredients. Secondly, contaminated ingredients and spillage in the storage bin also played a major role in persistent dissemination of a high contamination ratio. A contamination ratio of 70% was isolated from the storage bins and the storage houses on all poultry farms in our study. On the basis of previous studies, it can be predicted that this high ratio of contamination was a result of previously contaminated feed batches supplied to the farms or the remaining spillages in the feed bins.

Dust proved to be the most sensitive monitoring sample for the detection and isolation of *Salmonella* (Häggblom, 1994; Davis & Wray, 1997; Jones & Richardson, 2004). Samples from the cooler also showed a 75% contamination ratio. Most of the samples were collected as dust samples from the upper lids of the cooler, scrapings from the walls, upper lid and openings together with dust around the cooler areas. If this was a persistent contamination in the cooler coming from the dosing bin, then it was probably due to contamination passing though the pelleting with dust material that survived during the thermal process. I did not collect samples from the pellet machine because the thermal process was expected to eliminate all kinds of germs. This has been proved in previous studies (e.g. Himathongkham et al., 1996). However in feed mill B, *S. choleraesuis* was isolated from the dosing bin, the cooler as well as from the poultry farms. One could suspect that the warm, humid environment in the

cooler along with the presence of fatty feed deposits provided an ideal medium for the viability of the organism. It would have been more interesting to apply PCR test to see if it was the same serotype of *S. choleraesuis* which had been transferred from feed mill to the poultry farms. But the lack of time and funding made it difficult to go further, deeper into details.

A plausible gap remained during this study. Samples from the pellet making machine were not collected, assuming that all microbes are killed during the heating process. But for the future research, it would be highly recommended to investigate the effective role of temperature, humidity and time for complete elimination during this procedure. Furthermore, samples must also be taken from this compartment to investigate whether the contamination is passing to the next compartment through futile pellet making process or through dust particles and cross contamination.

Samples from Feed mill C, B, A and D resulted in 93.3%, 83.3%. 50% and 40% contamination rate respectively. On the basis of these results, it may be assumed that the environment of the mills is highly contaminated and most probably, free movement of dust is responsible for the dissemination of microbes from one compartment to all others.

Only *S. choleraesuis* and *S. gallinarium* were found in this study. Their presence indicated that several compartments in the feed mills were contaminated with *Salmonella*. Pellet making and the use of organic acid compounds did not guarantee that the feed would be free of all kinds of contaminations. Kirby and Davis (1990) studied that *S. typhimurium* could survive for 60 min at 100^{o}C in highly dehydrated conditions. Even if the heat treatment is effectively conducted, still there are risks of recontamination in the coolers. Moreover, feed millers in Pakistan are using 80^{o}C temperature for conditioning and pellet making (Anonymous, 2011) while data from the study of Jones and Richardson (2004) suggested that 85^{o}C temperature is required for feed to be *Salmonella* free.

Our sampling data for the assessment of *Salmonella* and ENT contamination in feed appeared to be quite low in numbers. Jones & Richardson (2004) found that *Salmonella* contamination is not uniformly distributed in the feed and to detect the level of contamination accurately, several hundred samples are required. However, they developed the link between ENT and *Salmonella* by logic, since *Salmonella* belongs to the same family. Mitchell & McCheseney (1991) suggested that in order to declare a batch of feed, *Salmonella* free, 30 samples must be analyzed individually. On the other hand, Häggblom (1994) found that large volumes of sampling material make it expensive to test *Salmonella* contamination. Furthermore, he suggested that sample collection only from feed manufacturing facilities including the raw material receiving pits, dust collection filters, top of pellet coolers, pellet cooling area and the top of finished feed bins is enough to isolate *Salmonella*. Since 1993, Sweden has abandoned the sampling of individual feed samples to detect the *Salmonella* contamination in poultry feed.

It was observed during the sample collection that there was a free movement of people between the milling compartments. There was no clear demarcation and restrictions for personnel movements. The mills were not using any kind of good manufacturing practices as every feed miller is bound to use in Sweden. During one visit to a Swedish feed mill, it was observed that they developed some restricted areas including coolers, where nobody was allowed to enter and the complete milling system was fully controlled or automatic. Furthermore, all compartments of the milling process were clearly demarcated from each other in order to control the cross contamination.

During the visits at the feed mills in Pakistan, it was observed that the storage areas were not well protected against birds and rodents. Davies and Wray (1997) suggested to the feed companies that samples from wild bird's droppings can be collected to identify the dangers of contamination of intake pits, warehouses and out loading gantries. Davies and Wales (2010) found more direct contamination by wildlife around feed mills. For instance, *S. enteritidis* was isolated in the flocks of laying hens, rodents and surroundings of feed mill. Furthermore, they isolated *S. typhimurium* which was widespread amongst samples from rodents, wild birds, stock, and the ingredient areas of another feed mill.

Recommendations to improve *Salmonella* situation

Lewerin et al. (2005) described the details of the effective control measures of *Salmonella* in Sweden. In the light of their studies, here are some suggestions which may be adopted for the control of *Salmonella*.

The implementation of a comprehensive control programme is needed to improve the *Salmonella* situation in the Pakistan poultry industry. The objective of a control programme is to provide *Salmonella* free meat and eggs to the consumer. The control programme should cover the whole production chain including poultry feed, breeders, hatchery, egg production, slaughter and processing. Street slaughtering which is still a common practice, should be controlled and moved to the modern slaughter houses. There is a need to establish a national policy which may describe the facts that all serotypes of *Salmonella* are pathogenic for humans. Other means of freedom from *Salmonella* can be used since the use of antibiotic is not the permanent solution. Sweden has also achieved low level of antibiotic resistance against salmonellas by avoiding the practice to use the antibiotics to treat *Salmonella* infection (SVARM, 2003).

For a successful *Salmonella* control programme, it is important to apply strict bio-security measures for breeder flocks. Most of the breeding companies in the world keep their pedigree flocks free from all serotypes of *Salmonella*. Wierup et al. (1995) mentioned in their studies that Sweden has euthanized 12 out of 39 imported breeding flocks, which were found to be *Salmonella* positive during 1982-1988. If contaminated feed has been supplied to the breeding flocks, there will be a continuous transmission of the microbes to the meat producing birds.

Policy

The policy should be based on an idea that the animals which are transported for slaughter are free from *Salmonella*. Furthermore, following strategies may be applied:

- Prevention of *Salmonella* contamination through high level of bio-security and hygiene control measures in the poultry production chain.

- Routine monitoring (of all commercial flocks and feed producing companies) at critical points through sampling and bacteriological cultural methods.

- Designing an immediate action plan: if any sample is detected positive for *Salmonella,* it must be reported to the veterinary authorities for further inspection and record.

What control measures are important to adopt

Here is a brief introduction for effective *Salmonella* control measures which may be adopted and implemented to improve *Salmonella* condition in the future.

- The government may build up legislations on the basis of regulations and guidelines for the control of *Salmonella* in animals and feed production. The poultry feed millers and farmers can be motivated for joining the voluntary control programme by adopting the hygiene control measures and regular sampling schedules. Furthermore, if any of the poultry flocks is found to be *Salmonella* positive, the eradication of the flocks may be compensated by the state insurance policy.

- The risks involved in poultry production are contaminated drinking water, feed, breeding stock, and husbandry practices. Large volumes of feed and water are consumed by the birds. For an effective *Salmonella* control programme, it is essential to identify the critical points all the way through the production chain. The feed mill may generate a certificate of contamination free feed for the farmers for every supply of a feed batch. The residual contamination in poultry houses must be free from *Salmonella* for the future flock which may be achieved by proper cleaning and disinfection methods. There are further chances of introduction of *Salmonella* infection during transportation. The transportation vehicles for eggs, meat, birds or feed must be cleaned and disinfected. Moreover, if the *Salmonella* is detected at any level, it must be possible to find the source of contamination within the entire production chain in order to stop the spread of the organism.

- The control of feed production may be based on application of the principles of Hazard Analysis Critical Control Point (HACCP). This technique can identify the main hazards in the processing line. The most important risk factor is raw material includ-

ing local and imported protein source meals. Most of the exotic serotypes of *Salmonella* are imported along with feed ingredients from other countries. All kinds of raw material that is found to be positive for *Salmonella* can be treated with organic acids before entering the mill vicinity. It is more important that, once the raw materials are free of contamination, care must be taken to avoid the re-contamination during the milling process and further during transportation and storage at the farm.

- Feed mills may be monitored by weekly sample collection and analyzed for *Salmonella*. The critical points for sample collection are unloading pits for raw material, air aspiration filters, top of pellet coolers, area surrounding the pellet coolers and top of the finished feed bins. Minimum 30 samples of dust and scrapings, less than 25 g, can be collected as samples. If *salmonella* is detected at any stage, the production should be stopped immediately and an extensive investigation should be carried out in the mill and the feed batches which are already delivered to the farms.

- The establishment of a professional rodent control programme is necessary and building should be sealed in order to prevent rodents and wild birds from gaining access. The holes, cracks, windows and ventilation apertures should be sealed with nets. Spillage around the silos should be immediately removed because it can attract rodents and wild birds.

- The area of feed mill can be divided into several restricted sections and zones. Free movement of people, working in several sections may be restricted to one specific section. Specially, mill workers may not be allowed to enter in the cooling zone during the milling process.

Conclusion

Researchers have been establishing links between poultry feed and Salmonellosis (both human and animal) for many years in the developed countries. There is a dire need to develop a better understanding and to assist poultry stakeholders to reduce *Salmonella* invasion in the poultry industry in developing countries, including Pakistan. Consequently, the risk involved in transferring *Salmonella* to humans will be decreased. This study also provided relevant information of factors that transmit the contamination, along with isolation and sampling methods for the feed milling industry.

The future perspective of this study is, to introduce the knowledge, to keep the whole chain of poultry production free from *Salmonella*. Regulations must be introduced and applied in order to control and prevent transmission of zoonotic *Salmonella* from animals to humans. Since *Salmonella* pathogenesis is complex, thermal process and pelleting may not be enough methods to eliminate the *Salmonella* completely from feed. Recontamination during cooling or transportation, unlimited mobility of people and animals within the feed mills are also of severe importance. There is still a need for advanced research in various areas of food safety. The ability of *Salmonella* to change the genome will result in new challenges for researchers to study antibiotic resistance genes, pathogenesis, virulence factors, clonal spread, environmental persistence and general epidemiology in the future.

Routine bacteriological supervision of the feed production in factories needs to be monitored. However, additional research is required to elucidate the practices which are associated with such high contamination rates and to reduce the burden of *salmonella* by controlling the pathogen in feed.

The challenge for the food industry in the future is, to produce high quality food and to meet high standards of animal welfare as practiced in countries as Sweden. Many aspects of animal production need to be considered in order to ensure the availability of cheap and safe food. When designing a control programme, it is important to keep a balance between human health, science, political perspective, economics and animal welfare.

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