Executive Summary

Title of the Project: Development of a single multiplex PCR for identification of mastitis causing microorganisms along with the antibiotic resistant genes against commonly used antibiotics in dairy animals

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Total Grant Allocated: 11, 60, 000/-

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Total Grant Utilized: 10, 06, 931/-

Objectives:

1. Isolation, identification and antibiogram of common bacterial/fungal pathogens leading to mastitis in and around Ludhiana, Punjab will be done using conventional method.
2. The common bacterial/fungal pathogens will be short listed according to prevalence and the antibiotic resistance pattern will be studied.
3. Appropriate primers for the organisms to be used in the multiplex will be designed from the genome available and a single Multiplex PCR having capability of identifying all the organisms simultaneously will be tried with the known bacterial strains.
4. Identification of antibiotic resistant genes from the knowledge of antibiogram for individual organism and primers for these will be designed that could be used in the multiplex too.
5. Multiplex against common antibiotic resistance genes in an organism will be developed and evaluated by testing in the raw milk samples and its diagnostic potential will be identified.

Summary of the Findings

Mastitis is the inflammation of the udder characterized by pathological changes in the mammary gland tissue. The most common treatment regimen involves administration of antibiotics depending upon culture and antibiotic sensitivity test. Culture and antibiotic sensitivity testing requires a minimum of 2-3 days, thus search for alternative tests to quicken identification of causative agent has been the focus of research. In mastitis, milk is the ideal sample for identification of causative agents as well as performing DNA based tests such as

PCR. Milk though easy to collect, harbor certain inhibitors affecting isolation of DNA and further when the same DNA is used for PCR these inhibitors affect PCR efficiency by interfering with PCR. In the present study a total of 102 milk samples from mastitic animals (clinical and subclinical) were collected that yielded 50 samples with bacterial growth. Out of these 50, 16 samples had single bacterial growth whereas rest of the 34 samples had mixed growth and thus a total of 43 *Staphylococcus*, 15 *Streptococcus*, 26 *E. coli* and 19 *Klebsiella* were isolated. All these isolates were screened for antibiotic sensitivity test against 20 antibiotics and chloramphenicol was found most sensitive followed by gentamicin, co-trimaxazole, ciprofloxacin, ofloxacin, gatifloxacin, cephalexin and sparfloxacin. From the results, it was also observed that the maximum antibiotic resistance was observed against penicillin followed by methicillin, amoxicillin and teicoplanin and streptomycin. Out of all the organisms isolated, *E. coli* revealed high sensitivity to chloramphenicol, co-trimoxazole, ofloxacin, cephalexin and gatifloxacin, *Klebsiella* was sensitive to chloramphenicol, co-trimoxazole, gentamicin, ciprofloxacin, doxycycline, *Staphylococcus* to chloramphenicol, gentamicin, ofloxacin, co-trimoxazole, ciprofloxacin, gatifloxacin, cephalexin and sparfloxacin, doxycycline and tetracycline whereas *Streptococcus* was sensitive to chloramphenicol, ciprofloxacin, sparfloxacin, co-trimoxazole, ofloxacin and gentamicin.

In the next experiment, bacterial DNA was extracted using 3 methods directly from culture (M1), directly from milk sample using Power Food Microbial DNA isolation Kit (M2) and SDS-Triton method (M3). Bacterial DNA was extracted from all the three methods and was subjected to PCR using genus specific primers as well as primers to identify antibiotic resistant genes. The results indicated that the detection of individual microorganism was best using Method 1 followed by Method 3 and Method 2. Bacterial DNA extracted using Method 1, Method 2 and Method 3 were subjected to PCR for detecting antibiotic resistant genes and were compared with the antibiotic sensitivity test results to identify the best method on the basis of successful amplification of antibiotic resistance genes. It was observed that when the results of Method 2 and 3 were compared with that of Method 1 and AST it revealed that using Method 3 we could get more positive PCR amplification of the antibiotic resistance genes than with Method 2.

In another experiment primers were designed for simultaneous identification of four most common organisms causing mastitis and a multiplex PCR was developed to identify and differentiate *Staphylococcus, Streptococcus, Klebsiella* and *E. coli* in a single reaction. Primers were standardized initially by using them individually against the DNA extracted from standard bacterial strains procured from MTCC, Chandigarh. Once the individual

primers gave the desired amplicon in an individual reaction we preceded for multiplexing. Multiplex PCR was found to be successful in identifying three organisms simultaneously viz., *Streptococcus, Klebsiella, E. coli* in a single reaction. Further MPCR was tested directly on the field samples (milk) and was found to successfully amplify desired product.

Finally, primers were redesigned for identification of genus and primers for antibiotic resistant genes were searched and those which could be used in MPCR were selected. Thus, a total of 16 primers were selected which could be used for MPCR. Since these resulted in spurious amplification when used altogether thus, two MPCR one incorporating gram positive organisms along with antibiotic resistant genes whereas other incorporating gram negative organisms along with antibiotic resistant genes was selected. When these MPCR was used on milk samples it was found that we could amplify genus as well as antibiotic resistant genes together.

Conclusions:

1. *Staphylococcus*, *Streptococcus*, *E. coli* and *Klebsiella* were most commonly isolated organisms from mastitis cases.
2. A modified method to isolate DNA from milk was standardized and tested.
3. A multiplex PCR that could identify *Streptococcus*, *Klebsiella* and *Escherichia coli* in a single reaction was developed.
4. Also, two MPCR one for identifying gram positive and one for identifying gram negative organisms along with their antibiotic resistant genes too were developed.

Contributions to the society

Control measures of mastitis in dairy cattle needs sensitive, rapid and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. The conventional methods for the identification of mastitis pathogens are labor intensive and time taking. Hence, relying on techniques using molecular tools for rapid, sensitive and specific identification of the etiological agents is one of the most important aspects for researchers. In mastitis, milk is the ideal sample for performing DNA based tests such as PCR. Milk though easy to collect, harbor certain inhibitors affecting isolation of DNA and further when the same DNA is used for PCR these inhibitors affect PCR efficiency by interfering with PCR. Thus, in the present study, a modified method for extraction of bacterial DNA directly from milk (SDS-Triton method) was developed. This method was found effective in extracting bacterial DNA from the milk directly and the extracted DNA was used to successfully amplify genus specific genes as well as the antibiotic resistance genes of the tested organisms. Further, a multiplex PCR capable of simultaneously identifying three organisms

i.e. *Streptococccus*, *Klebsiella* and *Escherichia coli* too was developed, standardized and tested on field samples. Thus using multiplex PCR we could identify the causative organism quickly and precisely. Also, two MPCR one for identifying gram positive and one for identifying gram negative organisms along with antibiotic resistant genes too was developed. Developing these MPCR too could save enormous time and money as these would tell not only the etiological agent leading to infection but antibiotics which would be resistant in those etiological agents.