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Microbiological monitoring of Rodent pathogens using Multiplex method of PCR

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Abstract

Microbiological quality control is undertaken to check the health status of laboratory animals. Pathogens infect the laboratory animals in various respiratory, digestive, central nervous systems, haematopoietic systems, dermal systems, etc. PCR detects pathogens with high sensitivity and specificity when compared to ELISA. Multiplex PCR can detect two or more pathogens at the same time. Multiplex PCR gives result based on the enzyme activity, bonding between the primer and DNA. In the present study, animal samples were analysed for the pathogen DNA at the molecular level to know the health status of laboratory animals. The respiratory pathogens *Mycoplasma pulmonis* and Sendai virus were detected simultaneously at 245, 340 bp, respectively. The digestive pathogens *Salmonella typhimurium* and *Clostridium piliforme* were detected simultaneously at 246, 196 bp, respectively. The animals were free from the specific respiratory and digestive pathogens. The multiplex PCR method is beneficial to detect the various pathogens in the laboratory rodents using the high throughput method.

Keywords: Microbiological monitoring, Rodent pathogens, Multiplex PCR.

Introduction

Microbiological quality control is performed to assess if the laboratory animals are free from bacteria, protozoa, yeast, fungi, viruses, rickettsia, mycoplasma and agents such as helminths and arthropods. Infection can be transmitted through contaminated bedding, water, food, air or infected animal facility personnel. Microscopic examination, cultural isolation and serology are the primary methods to detect viruses, bacteria, parasites. The new methodof molecular detection-PCR, ELISA made the process of pathogen detection easier (Shek et al., 2002).

Pathogens are commonly found in various systems of laboratory animals such as respiratory system, digestive system, dermal system, hematopoietic system, central nervous system, and other systems. Respiratory and digestive pathogens are the most critical pathogens which interfere with the experimental results (Baker, 1998).

Sendai virus and *Mycoplasma pulmonis* are the most important respiratory pathogens of laboratory animals. These infections are asymptomatic. It affects mice, rat, hamster and other laboratory animals. Sendai virus is a ssRNA virus of family *Paramyxoviridae*, genus *Paramyxovirus* and species *parainfluenza*. Sendai virus is highly contagious and transmitted through direct contact and aerosol of the respiratory tract. The clinical signs of Sendai virus infection include transient hypertrophy, necrosis, and repair of airway epithelium (Baker, 1998).

Mycoplasma pulmonis causes Murine Respiratory Mycoplasmosis (MRM) in laboratory animals. Transmission is primarily intra-uterine and also by aerosol. The organism establishes infection by nasopharynx and middle ears colonization. Clinical signs include snuffing in rats and chattering, dyspnea, weight loss, hunched posture in mice. *Mycoplasma pulmonis* infection alter the experimental results in ciliary function, cell kinetics, immune response etc. (Baker, 1998).

The most critical digestive pathogens of laboratory animals are *C. piliforme* and *Salmonella typhimurium. C. piliforme* (formerly known as *Bacillus piliformis*) causes Tyzzer's disease . *C. piliforme* is a Gram-negative, filamentous, endospore-forming bacterium. It is transmitted through ingestion of infectious endospores in contaminated food or bedding. Most of the infections are subclinical. Clinical signs include suckling and weanling in rodents, sudden death, watery diarrhea, lethargy, and ruffled fur. The disease is found to alter the experimental results in pharmacokinetics of warfarin and trimethoprim and the activity of hepatic transaminase (Baker, 1998).

Salmonella enteritidis is a Gram-negative, non-endospore forming bacteria and has roughly 1500 serotypes. It colonizes in the intestinal tracts of animal hosts. The most important serotype of *S. enteritidis* is *typhimurium*. It is transmitted through ingestion of contaminated feed, water, contaminated bedding and also through direct contact and animal facility personnel. The clinical signs include local hyperemia, focal necrosis, and pyogranulomatous inflammation consistent with septicemic disease (Baker, 1998).

The microbiological monitoring of parasites is performed by microscope through sedimentation method, floatation technique and tape test. Enzyme Linked Immunosorbent Assay (ELISA) is used for bacteria and virus detection. Polymerase Chain Reaction (PCR) is used for bacteria, parasites and virus detection. Among these methods, PCR is an effective technique for monitoring the pathogen at molecular level. PCR detects pathogens with high sensitivity and specificity when compared to ELISA. It detects the pathogen by the amplification of DNA in an exponential manner with the smaller amount of DNA (Rahman et al., 2013). Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction (Henegariu et al., 1997). Multiplex PCR is a simple, fast and convenient method for identification and detection of various pathogens simultaneously. It will be handy for the health status monitoring of large animal facilities with more laboratory animal strains. It can detect two or more pathogens simultaneously in a single reaction. Applications of multiplex PCR are most useful for pathogen identification, gender screening, linkage analysis, forensic studies, template quantitation and genetic disease diagnosis (Edwards and Gibbs, 1994).

The presence of rodent pathogen testing modality in the rodent facility helps identify the specific pathogen-free status of animal colonies. In this study, respiratory pathogen combination (*Mycoplasma pulmonis* and Sendai virus) and digestive pathogen combination (*C. piliforme* and *Salmonella typhimurium*) are detected simultaneously in a single reaction with the specific primers.

Material and Methods

The Institutional Animal Ethics Committee (IAEC) of ACTREC, Navi Mumbai, which is endorsed by the Comiittee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Governement of India, approved the proposal for the use of laboratory animals for quality control vide proposal no. 01/2021. The laboratory animals were maintained under controlled conditions of temperature, humidity, light, positive pressure, noise, and light and dark cycle https://actrec.gov.in/cri-research-support-facility-detail/70. Randomly selected healthy or moribund animals, two from each of the strain A/J, BALB/c, B6D2F1, C57BL/6, CD-1, C3H, DBA/2, FVB, ICRC, Swiss, Swiss/ba, Nude mice, SCID mice, SD rat and Golden hamster were sacrificed and dissected under aseptic conditions. For PCR study, samples such as caecum and lungs were collected from these 30 rodents from 15 various strains. They were stored at -80°C until further use. The lung samples were processed for DNA extraction, and caecum samples were processed for RNA and cDNA extraction.

DNA extraction

DNA was extracted from all lung samples by using phenol-chloroform method. In brief, the lung piece was digested overnight in 300 µl of digestion buffer containing 20 µl of proteinase K (20 mg/ml in 1X TE Buffer) at 50°C in a roller mixer incubator. The next day 200 µl digestion buffer was added and mixed thoroughly. An equal volume of Tris-saturated phenol was also added and mixed gently by inverting the tubes for at least 10 minutes. The tubes were centrifuged at 13416 g for 5 minutes using a cold centrifuge at room temperature. The aqueous (top) layer was transferred into a fresh Eppendorf tube. An equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) was added and mixed gently by inverting the tubes for 5 minutes and centrifuging at 13416 g for 5 minutes. The aqueous (top) layer was transferred into a fresh Eppendorf tube. An equal volume of chloroform:iso-amyl alcohol (24:1) was added and mixed gently by inverting the tubes for 5 minutes. The tubes were centrifuged at 19319 g for 5 minutes. The aqueous (top) layer was transferred into a fresh Eppendorf tube. An equal volume of chloroform was added and mixed gently by inverting the tubes for 5 minutes and centrifuged at 19319 g for 5 minutes. The aqueous (top) layer was transferred into a fresh Eppendorf tube. 3M sodium acetate 1/10th volume of aqueous layer was added, and isopropanol 7/10th volume of aqueous layer was added, mixed gently to allow DNA to clump, and incubated at room temperature for 10 minutes. The tubes were centrifuged at 19319 g for 5 minutes to pellet down the DNA. The supernatant was discarded, 70% ethanol was added and incubated for 10 minutes at room temperature. Finally, the tubes were centrifuged at 19319 g for 5 minutes to pellet down the DNA. The supernatant was discarded and DNA was air-dried at 37ºC or kept in an incubator. After complete drying, 50 µl of TE buffer was added to dissolve the

DNA (Ghatak et al., 2013). The concentration of DNA was determined by using Nano drop Spectrophotometer.

cDNA extraction

RNA was extracted with the help of RNA extraction kit (MB601) from HiMedia as per the manufacturer's instructions. Caecum tissues were homogenized in 1ml of RNA extraction reagent (100mg of tissue). The homogenized sample were incubated for 5 minutes at room temperature, centrifuged at 22673 g at 4°C for 10 minutes and transferred to a fresh tube. 200µl of chloroform was added per ml of RNA express reagent used, shaken vigorously for 15 seconds, and incubated for 10 minutes at room temperature. The mixture was centrifuged at 13000 rpm for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to a fresh tube, added with 500 µl of isopropyl alcohol, incubated at room temperature for 5 minutes and centrifuged at 22673 g for 10 minutes at 4°C. The supernatant was removed without disturbing the precipitated pellet on the sides and bottom of the tubes and the RNA pellet was washed with 1ml of ethanol (75%-100%). The sample was vortexed and centrifuged at 22673 g at 4°C for 5 minutes. The supernatant was discarded, the RNA pellet was dried for 5-10 min by air drying or vacuum, 50µl of DEPC water added, incubated at 55-60°C for 10-15 min. and stored at -80°C for the long term usage (Tan and Yiap, 2009).

The RNA was converted to cDNA by using Applied Biosystems (AB) kit. The PCR reaction containing 2µl of 10X RT buffer, 0.8 µl of 100mM DNTP, 2µl of 10X RT random primer, 1µl of reverse transcriptase, 4.2 µl of nuclease free water and 10 µl of RNA was set up at 27°C for 10 minutes and annealing temperature of 37°C for 2 hours, 85°C for 5 minutes and hold at 4°C. The resultant product of cDNA was used as a template for setting up a PCR reaction.

PCR amplification

For respiratory system pathogens, a combination of *Mycoplasma pulmonis* and Sendai virus was selected and standardized. For this, DNA was amplified in 16µl of a PCR mixture containing 8µl of 2X PCR Taq mix (SRL, 1X concentration), Taq mix 2X (SRL) having 20mM of Tris-HCl, 100mM Kcl, 3mM $MgCl_2$, 400 µM DNTP, 0.1U/µl of Taq DNA polymerase, 0.5 µl of each primer (0.4µm concentration), 4 µl of nuclease-free water and 2 µl of template DNA (50 ng). The primers supplied by Sigma and used in this study are listed in Table 1. To determine optimal annealing temperature for the multiplex PCR, a temperature gradient experiment was performed from 64 to 71°C. The cycling conditions included an initial denaturation for 5 min at 94°C, 34 cycles of 20 sec at 94°C, 30 sec at 71°C, 20 sec at 72°C and final extension for 3 min. All PCR products were separated on 2% gel stained with ethidium bromide.

For digestive system pathogens, a combination of *Salmonella typhimurium* and *C. pilifome* were selected and standardized. For this, cDNA was amplified in 16µl of a PCR mixture containing 8µl of 2XPCR Taq mix (SRL, 1X concentration), Taq mix 2X (SRL) containing 20mM of Tris-HCl, 100mM KCl, 3mM $MgCl_2$, 400 µM DNTP, 0.1U/µl of Taq DNA polymerase, 0.5 µl of *Salmonella typhimurium* primer (0.4µm concentration), 1µl of *C. pilifome* primer (0.8µm concentration), 3 µl of nuclease-free water and 2 µl of template cDNA (100 ng). The primers used in this study are listed in Table 1. To determine optimal annealing temperature for the multiplex PCR, a temperature gradient experiment was performed from 58 to 69°C. The cycling conditions included an initial denaturation for 5 min at 94°C, 34 cycles of 20 sec at 94°C, 30 sec at 69°C, 20 sec at 72°C, and final extension for 3 min. All PCR products were separated on 2% gel stained with ethidium bromide.

All positive control DNA/ cDNA used in this study were procured from the Central Institute for Experimental Animals (CIAE), ICLAS Monitoring Centre, Kawasaki, Japan.

Table 1: List of rodent pathogens and primers used in this study.

S. no.	Rodent pathogens	Sequence	Product sizes
1	*C. piliforme* (Furukawa et al., 2002)	Forward: GTGCTAGGTGTTGGGAAG	196
		Reverse: TACTTTACGTAGCCTGTCAA	
2	*Salmonella typhimurium* (Jeong et al., 2011)	Forward: ATTAATTATGGAAGCGCTCGCATT	246
		Reverse: GTAATGAGATCCATCAAATTAGCG	
3	Sendai virus Liu et al., 2001)	Forward: CGGGATCCAGACCCTTTGCTTTGC	340
		Reverse: ATTTGACATCGGCGTTTACTCCG	
4	*Mycoplasma pulmonis* (Kazuo et al., 1994)	Forward: AACAGCAGCTGATAATCAAA	245
		Reverse: CTGAAAGTTTTGAAGAGTTT	

Result and Discussion

ne administrations in New Zealand White rabbit. As it was radiolabelled, it was possible to identify the location and clearance patteren of the formulation In the present study, 30 lungs and caecum samples were aseptically collected from 13 different laboratory mice strains and one each rat and hamster strain. The laboratory animal strains are namely A/J, BALB/c, C3H/J, CD-1, C57BL/6, DBA/2, FVB, ICRC, Swiss, Swiss/ba, BDF1, Nude, NOD SCID, SD rat, and Golden hamster. The samples were stored at -80°C until further use. The PCR reaction was set up to detect respiratory system pathogens (*Sendai virus and Mycoplasma pulmonis*) and digestive system pathogens (*C. piliforme* and *Salmonella typhimurium*). Two duplex/multiplex PCR combinations were used for the simultaneous detection of these four pathogens. Initially, standardization of duplex PCR was done with 2-4 samples to check whether it gives relevant results or not, and then all thirty samples were tested. The use of gradient PCR was beneficial for the standardization of duplex PCR.

Specificity and sensitivity of singleplex PCR

The specific primers for the *Salmonella typhimurium, Clostridium piliforme* (Tyzzer's disease*), Mycoplasma pulmonis and* Sendai virus were used for the PCR amplification. The *Salmonella typhimurium* and *C. piliforme* were amplified at 246, 196 bp, respectively, without any cross-amplification of other digestive pathogens (Figures 1 and 2, respectively). *Mycoplasma pulmonis and* Sendai virus were amplified at 245, 340 bp regions, respectively, without any cross-amplification of other respiratory pathogens (Figures 3 and 4, respectively).

Specificity and sensitivity of multiplex PCR

The specific primers for the Sendai virus and *Mycoplasma pulmonis* were used and it resulted in a 340 and 245 bp product size, respectively, as amplified regions simultaneously (Figures 5 and 6) without cross-amplification of other pathogens. The specific primer for the *Salmonella typhimurium, Clostridium piliforme* (Tyzzer's disease*)* was used to determine multiplex PCR specificity. The simultaneous detection of *Salmonella typhimurium, Clostridium piliforme* (Tyzzer's disease*)* was performed using a specific primer for the amplification. It shows 246 and 196 bp, respectively, without any cross-amplification of other pathogens (Figure 7 and 8).

Although conventional methods of disease diagnosis are the gold standards for the diagnosis of microbial diseases, molecular techniques are used to precisely check the microbiological health of animal or disease diagnoses. Conventional methods like agar plate exposure, biochemical media tests, microscopy, and histopathology are generally used for the diagnosis of bacterial disease. Viral diseases are challenging to diagnose by these methods. They need either ELISA, PCR, IHC, or electron microscopy methods to diagnose as they cannot be cultured easily. Ecto- and endo-parasites are diagnosed with skin scrapping, sedimentation, floatation, or cellophane tape method. However, molecular/PCR techniques are also available for the diagnosis of endo-parasites. LAF, ACTREC have been using conventional, biochemical media tests, microscopy, histopathology, PCR, IHC, and electron microscopy method to diagnose some of the diseases in the animals maintained in the LAF (Ingle et al., 2011; Ingle, 1999; Ingle and Shinde, 2014; Ingle and Shinde, 2011; Shinde and Ingle, 2015).

Molecular diagnostic methods are sensitive, reliable, requiring less volume of sample and detect minor variation in the sample. The basic molecular tools to diagnose the disease in laboratory animals are PCR and PCR allied techniques such as PCR-RFLP, RT-PCR, PCR-ELISA, RRT-PCR, LUX-RRT-PCR, real-time PCR. PCR permits the specific sequence to amplify. After the PCR reaction, the PCR products can be examined with ELISA, RFLP, beat-based multiplex assay, High Resolution Melting (HRM) curve analysis, hybridisation, Loop-mediated isothermal amplification (LAMP), Nucleic Acid Sequence-Based Amplification (NASBA), nucleotide sequencing, phylogenetic analysis to check the rapidity and reliability of the pathogens (Parel et al., 2008; Hamada et al., 2018; Hofler et al., 2014). Multiplex PCR is a variant of conventional PCR, making it easier to diagnose various pathogens in a single reaction. a reporter DNA molecule is used in immuno-PCR (Dhama et al., 2012). *Mycoplasma pulmonis* and Sendai virus are the digestive system pathogens, and *Salmonella typhimurium* and *C. piliforme* are respiratory system pathogens. In the multiplex PCR reaction of Sendai virus and *Mycoplasma pulmonis,* both the DNA and primers were taken in a single tube and various samples were taken in different tubes with both the primers. There was no cross amplification of other pathogens and non-specific bands in the samples. In the multiplex PCR reaction of *Salmonella typhimurium* and *C. piliforme* both the cDNA and primers were taken in the single tube and various samples taken in different tubes with both the primers. There was no cross amplification of other pathogens and non-specific bands in the samples.

Based on the results of the study, all the samples were free from respiratory as well as digestive rodent pathogens tested. In this study, we have successfully devised a method to test the combinations of Sendai-*Mycoplasma pulmonis;* and *C. piliforme-Salmonella typhimurium* simultaneously. We should use the required primer concentration and optimum temperature condition for multiplex PCR reactions to express the expected bands. The limitation of multiplex PCR is that it cannot differentiate the pathogen with very close product size and must have a minimum difference of 25- 50 bp product size amongst the pathogens, for better demarcation after a long run. Compared to singleplex PCR, it also has the disadvantage of lack of detection of a single target gene and less detection sensitivity (Maria and Ribeiro, 2019)

Conclusion

Multiplex PCR is simple, fast, sensitive, and specific, and it can simultaneously detect multiple pathogens. This method is handy, high throughput, and is fast to screen the microbiological health status of the laboratory animal population. All animal

strains in the facility were found negative for the tested pathogens indicating stringent managemental practices. This helps us to declare that our rodent colony is a specific pathogen-free (SPF) for these pathogens by using minimum gel slabs.

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Figure 1: Gel image of singleplex PCR for *Salmonella typhimurium* detection. Lane 1- Negative control; lane 2- Positive control of *Salmonella typhimurium* (246 bp); lane 3- 100bp DNA marker; lane 4- cDNA from A/J; lane 5- cDNA from BALB/c; lane 6- Positive control of *Salmonella typhimurium* (246 bp).

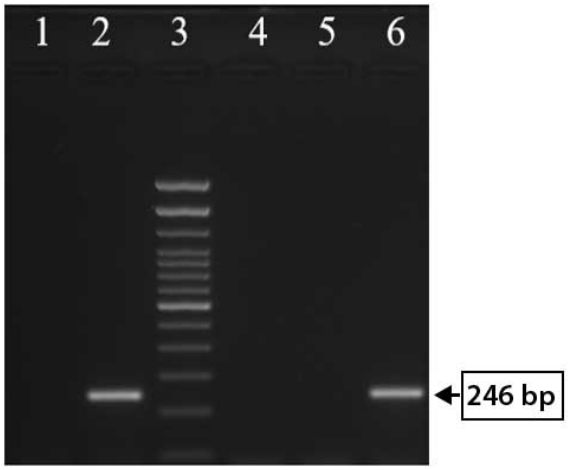

Figure 2: Gel image of singleplex PCR for *C. piliforme* detection. Lane 1- Negative control; lane 2- Positive control for *C. piliforme* (196 bp); lane 3- 100bp DNA marker; lane 4- Caecal cDNA from A/J; lane 5- Caecal cDNA from BALB/c; lane 6- Positive control for *C. piliforme* (196 bp).

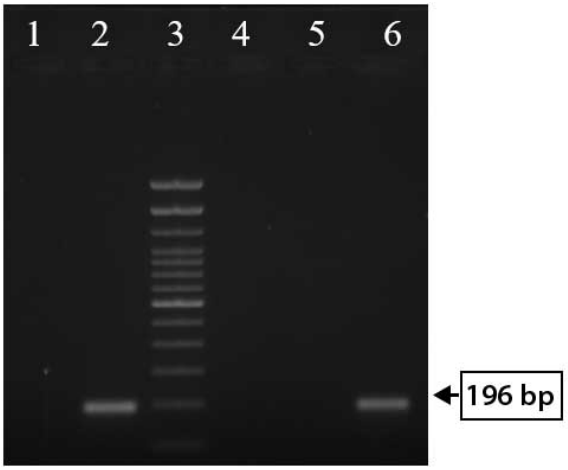

Figure 3: Gel image of singleplex PCR for *Mycoplasma pulmonis* detection. Lane 1- Negative control; lane 2- Positive control for *Mycoplasma pulmonis* (245 bp); lane 3- 100bp DNA marker; lane 4- Lungs DNA from A/J; lane 5- Lungs DNA from BALB/c; lane 6- Positive control for *Mycoplasma pulmonis* (245 bp).

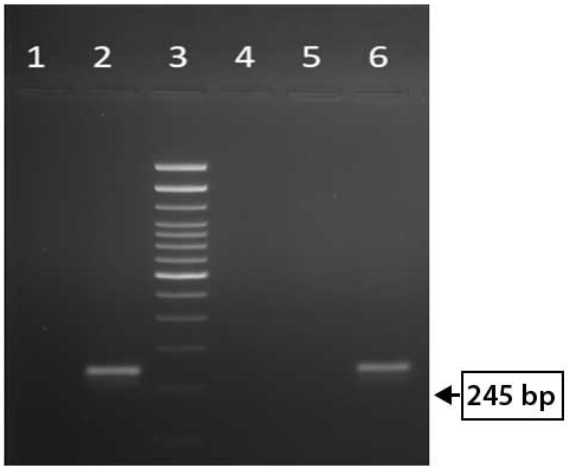

Figure 4: Gel image of singleplex PCR for Sendai virus detection. Lane 1- Negative control; lane 2- Positive control for Sendai virus (340 bp); lane 3- 100bp DNA marker; lane 4- Lungs DNA from A/J; lane 5- Lungs DNA from BALB/c; lane 6- Positive control for Sendai virus (340 bp).

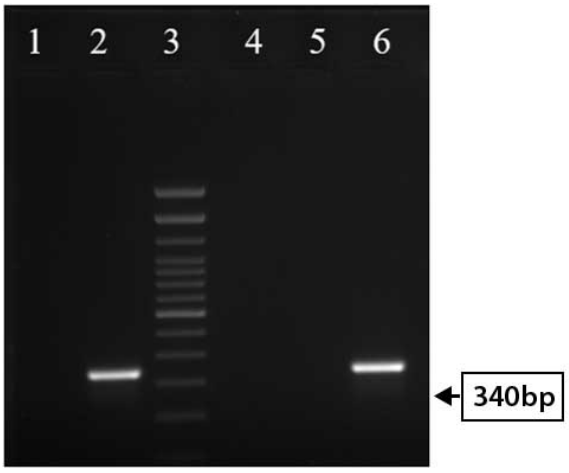

Figure 5: Gel image of multiplex PCR for Sendai virus and Mycoplasma pulmonis detection. Lane 1- Negative control; lane 2- Duplex positive control of Sendai virus and Mycoplasma pulmonis (340, 245 bp, resp.); lane 3- 16: Lungs DNA from A/J; lane 4- A/J; lane 5- BALB/c; lane 6- BALB/c; lane 7- C3H/J; lane 8-C3H/J; lane 9- CD1; lane 10- CD1; lane 11- C57BL/6; lane 12- DBA/2; lane 13- DBA/2; lane 14- FVB/NJ; lane 15- ICRC; lane 16- ICRC; lane 17- 100 bp DNA marker.

Figure 6: Gel image of multiplex PCR for Sendai virus and Mycoplasma pulmonis detection. Lane 17- 100 bp DNA marker; lane 18- 30: Lungs DNA from Swiss; lane 19- Swiss; lane 20- Swiss/ba; lane 21- Swiss/ba; lane 22- BDF1; lane 23- BDF1; lane 24- Nude; lane 25- Nude; lane 26- NOD-SCID; lane 27- NOD-SCID; lane 28- SD rat; lane 29- Golden hamster; lane 30- Golden hamster; lane 31- Duplex positive control of Sendai virus and Mycoplasma pulmonis (340, 245 bp, resp.).

Figure 7: Gel image of multiplex PCR for *C. piliforme* and *Salmonella typhimurium* detection. Lane 1- Negative control; lane 2- Duplex positive control of *C. piliforme* and *Salmonella typhimurium* (196, 246 bp, resp.); lane 3- 16: Caecal cDNA from A/J; lane 4- A/J; lane 5- BALB/c; lane 6- BALB/c; lane 7- C3H/J; lane 8- C3H/J; lane 9- CD1; lane 10- CD1; lane 11- C57BL/6; lane 12- C57BL/6; lane 13- DBA/2; lane 14- DBA/2; lane 15- FVB; lane 16- FVB; lane 17- 100 bp DNA marker.

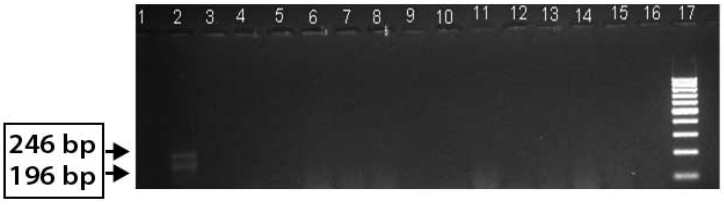

Figure 8: Gel image of multiplex PCR for *C. piliforme* and *Salmonella typhimurium* detection. Lane 17- 100 bp DNA marker; lane 18- 32: Caecal cDNA from ICRC; lane 19- ICRC; lane 20- Swiss; lane 21- Swiss; lane 22- Swiss/ba; lane 23- Swiss/ba; lane 24- BDF1; lane 25- BDF1; lane 26- Nude; lane 27- Nude; lane 28- NOD-SCID; lane 29- NOD-SCID; lane 30- SD rat; lane 31- SD rat; lane 32- Golden hamster; lane 33- *C. piliforme* and *Salmonella typhimurium* (196, 246 bp, respectively).