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A multiplex PCR for Massachusetts and Arkansas serotypes of infectious bronchitis virus

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Infectious bronchitis virus (IBV), the prototype of the coronavirus family, is an enveloped, single-stranded RNA virus with a genome size of approximately 27·6 kilobase. Infectious bronchitis virus causes an acute, highly contagious respiratory and urogenital disease of chickens which results in significant economic losses in commercial broilers, layers and breeders. A rapid, highly sensitive and specific method is needed in the differential diagnosis of infections of different serotypes. A multiplex polymerase chain reaction (PCR) method was developed and optimized to simultaneously detect Massachusetts (Mass) and Arkansas (Ark) serotypes of IBV. One common primer and two serotype specific primers were chosen from the S1 gene sequences of IBV and used in one PCR reaction. Under optimized PCR conditions, two serotype specific PCR products, 1026 bp for Mass and 896 bp for Ark, respectively, were amplified and detected by agarose gel electrophoreses. The specificity of the technique was verified by using 20 different strains and isolates of IBV, and other avian bacterial and viral pathogens. Using a serial 10-fold dilution of the artificial mixture of both Mass and Ark samples, the detection limit was found to be 5 pg RNA after 35 cycles of PCR. The multiplex PCR was able to detect and differentiate both serotypes in embryonated eggs that were co-infected with different EID₅₀ virus titers of Mass 41 and Ark 99. The multiplex PCR developed in this study will be valuable for rapid identification, differential diagnosis, and epidemiological studies of these two serotypes of IBV infections.

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INTRODUCTION

Infectious bronchitis virus (IBV), a member of Coronaviridae, is a positive sense, single-stranded RNA virus with a genome size about 27·6 kilobase. There are three structural proteins encoded in its genome, surface glycoprotein (S), membrane protein (M) and nucleocapsid protein (N). The S protein is cleaved into S1 and S2 during viral maturation. The S1 protein is responsible for the virus neutralization (VN), haemagglutination inhibition (HI) and serotype specific antibodies.¹ Infectious bronchitis virus infection is an

acute, highly contagious respiratory and urogenital disease of chickens that causes significant economic losses.² The weight gain, feed efficiency, egg production and egg quality are compromised in diseased flocks. In spite of vaccination, there has been an increase in the prevalence of IBV infections due to new serotypes or variant strains, especially in commercial breeders, broilers and layers.^{3,4}

Diagnosis of IBV infection is accomplished by isolating and serotyping the causative field isolate. The

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virus neutralization and haemagglutination inhibition tests have been used for serotyping.^{1,5} These procedures are expensive, tedious, time-consuming and not widely available to the poultry industry. Serodiagnosis of IBV using serum from recovered flocks cannot be used to identify the causative serotypes of IBV because chicken produce cross-reacting antibodies following multiple infections.⁶ Recently developed molecular methods such as monoclonal antibodies,⁷ polymerase chain reaction (PCR),⁸ reverse transcriptase-PCR-restriction fragment length polymorphism (RT-PCR-RFLP)^{9,10} and serotype specific RT-PCR¹¹ have been used for diagnosis and serotyping IBV infections. While useful, these techniques require multiple steps to identify a particular serotype. Simultaneous detection and differentiation of bacterial,^{12–14} mycoplasmal¹⁵ and viral¹⁶ pathogens have been described using multiplex PCR amplification techniques.

The present study describes the development and optimization of a multiplex PCR to detect and differentiate two important serotypes of IBV in a single RT-PCR reaction.

MATERIALS AND METHODS

Virus and culture conditions

Table 1 shows a list of IBV strains representing six serotypes and three variants, and other avian pathogens and their sources. The IBV and Newcastle Disease Virus (NDV) were propagated in 11-day-old specific-pathogen-free (SPF) embryonated eggs. The allantoic fluids were harvested after 36 h of incubation at 37°C. Avian reovirus was propagated in chicken embryo fibroblasts and plaque purified as described previously.¹⁷ *Mycoplasma gallisepticum* S6 was grown in Frey's media.¹⁸

Extraction of RNAs and DNAs

Allantoic fluids from embryonated eggs infected with IBV, NDV and cell culture of reovirus were first clarified by centrifugation at 500 × g for 15 min. Supernatants were transferred to ultracentrifuge tubes. The virion pellets were obtained by centrifugation at 93 000 × g at 4°C for 3 h. The pellets were then resuspended in 1 ml of diethylpyrocarbonate (DEPC)-treated distilled water, followed by the rapid extraction of RNA using Trizol-LS reagent (Life Technologies, Bethesda, MD, USA) according to the guanidinium thiocyanate method.¹⁹ Chromosomal

DNA from *M. gallisepticum* S6 was extracted and purified by a previously described method.¹⁵

Primers selection

Alignment of the S1 gene sequences was performed on the North American IBV serotypes/isolates which include Mass 41,²⁰ Ark 99, PP14, SE-17,²¹ JMK, Gray,²² Holte (Genebank L18988), Conn²³ and Beaudette,²⁴ to identify the conserved and variable regions design primers. Serotype specific primers were chosen from a hypervariable region close to the N-terminus of S1 supposed to be associated with serotypes as previously described.^{1,25} One common lower primer was selected from the conserved C-terminus of S1 gene sequences. Primer sequences were analysed for secondary structure formation, G+C content, primer dimer formation, hairpin formation and their compatibility in the multiplex PCR using the OLIGO primer analysis software and MacDnasis, BESTFIT and BLAST programs²⁶ (Biotechnology Center, University of Connecticut, Storrs, CT, USA).

The common lower primer is a 24-mer (5' CGCTCTT AGTA ACA TAAA CTA ACA 3') sequence located at nucleotide position 1272–1296 in the Mass 41²⁰ and 1336–1360 in the Ark 99²¹ of S1 gene. The Mass specific upper primer (5' CTGGGTG TATTGTT GGTACTA TTC 3') is located at 270–294 nucleotide²⁰ and the Ark specific primer (5' ACATTGT TTTAAGA GCGGATCT AA 3') is located at the 464–488 position²¹ of the S1 gene. All primers were synthesized by the Biotechnology Center, at the University of Connecticut.

Reverse transcription (RT) and multiplex PCR reaction

The reverse transcription reaction was conducted using GeneAmp PCR Kit (Perkin Elmer Cetus, Norwalk, CT, USA). The reaction contained 4 µl of 80 mM MgCl₂, 2 µl of 10 × PCR buffer [500 mM KCl, 200 mM Tris HCl (pH 8.4), 0.5 mg ml⁻¹ nuclease-free bovine serum albumin], 2 µl of 10 mM each dinucleoside triphosphate (dNTP), 1 µl (20 units) of RNase inhibitor, 1 µl (0.5 µM) Random Hexamers, 1 µl (50 units) of Moloney Murine Leukemia Virus (MuLV) reverse transcriptase and 50 ng of RNA. A total volume of 20 µl reaction was obtained by adding (DEPC)-treated distilled water. The RT was conducted using a thermal cycler setting of 42°C for 15 min, 99°C for 5 min and 5°C for 5 min for one cycle.

For the multiplex PCR reaction, 6 µl of 80 mM MgCl₂, 8 µl of 10 × PCR buffer [500 mM KCl, 200 mM

Table 1. Avian pathogens used in the study

Isolate designation	Serotype/strain		Source
Mass 41	Mass	Reference virus	University of Connecticut
Mass	Mass		SPAFAS, Inc.
Mass 1	Mass		University of Connecticut
Mass 3	Mass		University of Connecticut
Mass 5	Mass		University of Connecticut
Ark 99	Ark	Reference virus	University of Connecticut
Ark c	Ark		University of Connecticut
Ark (SPAFAS)	Ark		SPAFAS, Inc.
Conn (SPAFAS)	Conn		SPAFAS, Inc.
Conn	Conn		University of Connecticut
33-CT	Variant/Conn		University of Delaware
92-174	Variant		University of Delaware
Fla	Fla	Reference virus	University of Delaware
CA-1 (Kinde)	Variant		University of Delaware
SE-17	Se-17	Reference virus	University of Delaware
JMK	JMK	Reference virus	University of Connecticut
JMK	JMK		University of Connecticut
Field Isolate 97:7852	Mass		University of Connecticut
Field Isolate 97:7853	Mass		University of Connecticut
Field Isolate 97:7854	Ark		University of Connecticut
Newcastle Disease Virus	B-1	Reference virus	Washington State University
Reovirus	S1133	Reference strain	University of Connecticut
<i>Mycoplasma gallisepticum</i>	S6	Reference strain	University of California

Tris HCl (pH 8.4), 0.5 mg ml⁻¹ nuclease-free bovine serum albumin], 1200 ng of common lower primer (500 ng μ l⁻¹), 465 ng of Ark primer (465 ng μ l⁻¹) and 347 ng of Mass primer (347 ng μ l⁻¹), 2.5 units of AmpliTaq DNA Polymerase were added in the above RT reaction tubes and 100 μ l of total volume was obtained by adding DEPC-treated distilled water. Multiplex PCR was performed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min.

Detection of amplified DNAs

Gel electrophoresis was used to detect amplified DNA products. A volume of 15 μ l of amplified PCR products was subjected to electrophoresis at 80 V in horizontal

gels containing 2% agarose (Ultrapure; Bethesda Research Laboratories, Bethesda, MD, USA) with Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA). The gel was stained with ethidium bromide (0.5 gm ml⁻¹), exposed to u.v. light to visualize the amplified products, and photographed.

The specificity and sensitivity of the multiplex PCR

To determine the specificity of the technique, 20 different strains of IBV, NDV, Reovirus and *M. gallisepticum* S6 as listed in Table 1 were examined. To determine the capability of the multiplex PCR technique to detect and differentiate Mass and Ark IBV serotypes in the same reaction, 11-day-old embryonated eggs were inoculated with three different

EID₅₀ combinations of both Mass 41 and Ark 99, which included Mass 41 10^{5.3} with Ark 99 10^{4.2}, Mass 41 10^{3.3} with Ark 99 10^{4.2}, and Mass 41 10^{2.3} with Ark 99 10^{4.2}. Allantoic fluids were harvested after 36 h incubation at 37°C. Total RNA was extracted as previously described and 50 ng from each allantoic fluid were used as template RNA. To determine the sensitivity of the multiplex PCR, a serial 10-fold dilution of the artificial mixture of 50 ng of individual Mass and Ark were used as template RNAs.

Virus neutralization (VN)

The VN test was performed in 10-day-old SPF embryonated eggs as described by Gelb *et al.*⁵ Briefly, equal parts of a serial of virus dilutions and 10⁻¹ serum dilution in tryptose phosphate buffer (TPB) were mixed and allowed to react at room temperature for 60 min. Normal serum controls were prepared in TPB at dilution equivalent to the serum concentration used. Embryos inoculated with the serum virus mixtures were examined for IBV typical lesions (stunting, curling and kidney urates) 7 days post-inoculation. Reference viruses used were Mass, Ark and JMK. The 50% neutralization end points were calculated as described by Reed and Muench.²⁷

RESULTS

An IBV specific multiplex PCR amplification technique was optimized which would be able to identify Mass and Ark serotypes of IBV in a single RT-PCR reaction. The IBV multiplex PCR products consisted of 1026 bp for IBV-Mass and 896 bp of IBV-Ark serotypes as expected and there were no detectable DNA bands observed for the other IBV strains or serotypes (Fig. 1). No spurious PCR amplification reactions between these two serotypes were observed. There were no PCR products generated from the NDV, avian Reovirus and *M. gallisepticum* S6 nucleic acid (data not shown). The experiment was repeated three times and the results were reproducible. The multiplex PCR assay developed and evaluated in this study was found to be a specific assay for IBV Mass and Ark serotypes.

The multiplex PCR was able to detect RNAs of these two IBV serotypes at levels as low as 5 pg (Fig. 2). No spurious PCR amplification between these two serotypes were observed using various amounts of both RNA mixtures. In addition, the amounts of the amplified products showed a linear correlation to the amount of RNAs in the dilution. Using the multiplex PCR, both IBV-Mass and IBV-Ark were detected in

three individual mixed infection samples of Mass and Ark from the co-infection experiments conducted in the embryonated eggs (Fig. 3). From the density of the bands, the relative amounts of Mass and Ark virion RNAs can be estimated and are consistent with the titer of each serotype used in the co-infection experiments. Both the VN test and the multiplex PCR technique were used to identify three IBV isolates from recent outbreaks. The VN test results indicated that two of the isolates were Mass serotypes, one was Ark serotype, and the multiplex PCR results agreed with that of the virus neutralization test (data not shown).

DISCUSSION

The RT-PCR-RFLP methods described by Kwon *et al.*⁹ and Lin *et al.*¹⁰ are capable of identifying different serotypes of IBV. However, usually more procedural steps are required to identify the PCR product that makes such tests somewhat cumbersome and time-consuming. Most recently, the serotype specific RT-PCR developed by Keeler *et al.*¹¹ greatly improved the speed of serotyping and diagnosis of IBV, but it still needs to conduct multiple individual RT-PCR tests to identify a serotype. A Mass and Ark specific multiplex PCR technique has been developed which has the advantage of rapid identification of multiple serotypes of IBV. It also saves time and money in comparison to conducting each individual PCR.

Sequence analysis of different serotypes of IBV S1 gene suggested that a few amino acids change could give rise to a new serotype, particularly if it occurs in an area close to the N-terminus of S1 gene.^{1,25} Most of the nucleotide mismatches are concentrated in the N-terminus of S1 gene, few are found in the C-terminus of S1 gene. Kwok *et al.*²⁸ demonstrated that two to three mismatches at the end of 3' of primers can be specific and can be applied in the design of strain-specific primers. In this study, one common lower primer was selected from the C-terminus and two serotype specific upper primers from the N-terminus by comparing the known S1 sequences of different serotypes or strains from North America to use in multiplex PCR.

Multiple mismatches at the 3' end of the Ark upper primer exist when it is aligned with Mass sequence. There is one mismatch at the 3' end of Mass primer when it is aligned with the Ark sequence, but eight out of the 24 nucleotides are different. Initially, a low annealing temperature 50°C was used. All four strains of Mass serotype yielded a strong specific PCR product corresponding to 1026 bp. All three strains of Ark serotype generated specific PCR product about

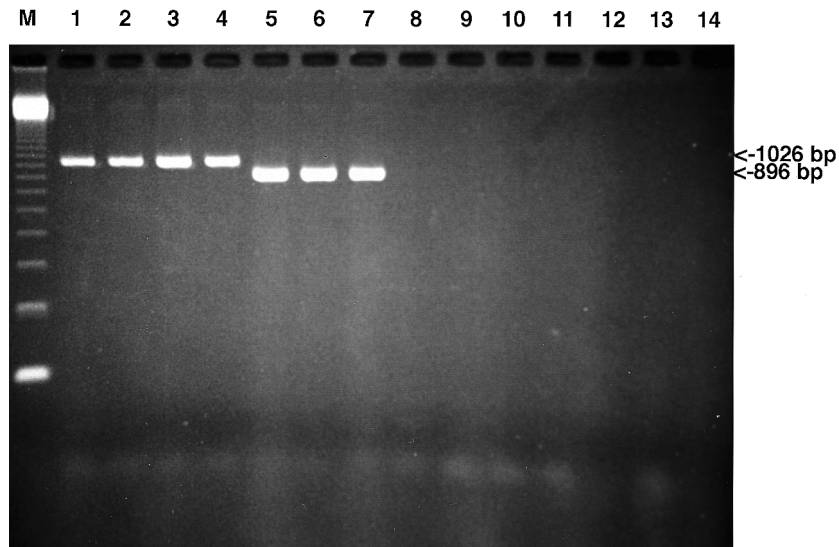


Fig. 1. Agarose gel electrophoresis of infectious bronchitis virus (IBV) specific cDNA products amplified by reverse transcriptase-multiplex polymerase chain reaction. Lane M=Molecular size marker (123 base pair DNA Ladder); Lane 1 =IBV-Mass 41; Lane 2 =IBV-Mass (SPAFAS); Lane 3 =IBV-Mass 1 field; Lane 4 =Mass 5 field; Lane 5 =IBV-Ark 99; Lane 6 =IBV-Ark (SPAFAS); Lane 7 = IBV-Ark c; Lane 8 =IBV-Conn; Lane 9 =IBV-JMK; Lane 10 =IBV-33-CT; Lane 11 =IBV-Field 92-174; Lane 12 =IBV-Florida; Lane 13 =IBV-CA-1; Lane 14 =IBV-SE-17.

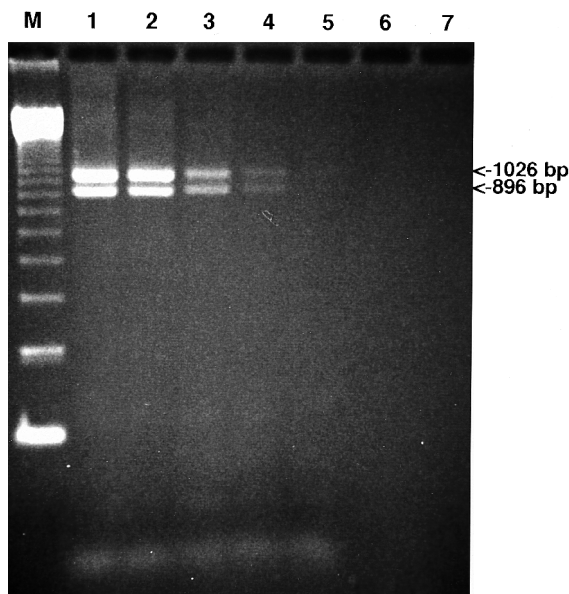


Fig. 2. Sensitivity of reverse transcriptase-multiplex polymerase chain reaction. Lane M=Molecular size marker (123 base pair DNA Ladder); Lane 1 =50 ng of each infectious bronchitis virus (IBV)-Mass 41 and IBV-Ark 99 RNA; Lane 2 =5 ng of each IBV-Mass 41 and IBV-Ark 99 RNA; Lane 3 =500 pg of each IBV-Mass 41 and IBV-Ark 99 RNA; Lane 4 =50 pg of each IBV-Mass 41 and IBV-Ark 99 RNA; Lane 5 =5 pg of each IBV-Mass 41 and IBV-Ark 99 RNA; Lane 6 =500 fg of each IBV-Mass 41 and IBV-Ark 99 RNA; Lane 7 =50 fg of each IBV-Mass 41 and IBV-Ark 99 RNA.

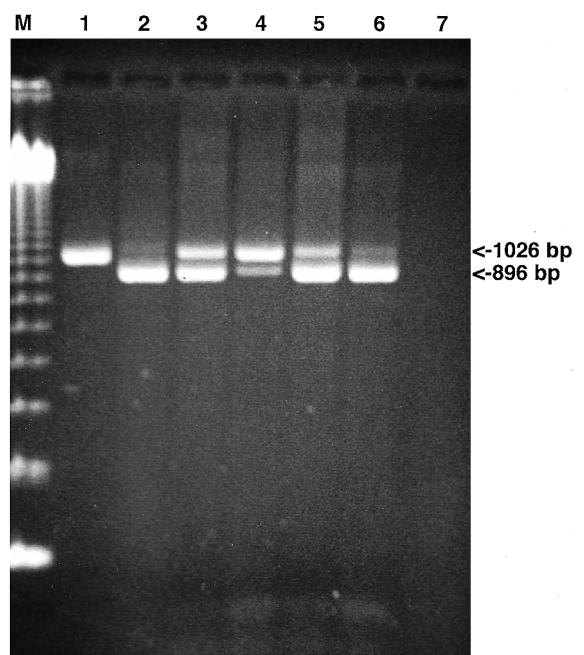


Fig. 3. Agarose gel electrophoresis of infectious bronchitis virus (IBV) specific cDNA products amplified by reverse transcriptase-multiplex polymerase chain reaction to detect the Mass 41 and Ark 99 in mixed infection samples. Lane M=Molecular size marker (123 base pair DNA Ladder); Lane 1 =Mass 41; Lane 2 = Ark 99; Lane 3 =Mixture of Mass 41 RNA and Ark 99 RNA; Lane 4 =Co-infection of Mass 41 $10^{5.3}$ with Ark 99 $10^{4.2}$; Lane 5 =Co-infection of Mass 41 $10^{3.3}$ with Ark 99 $10^{4.2}$; Lane 6 =Co-infection of Mass 41 $10^{2.3}$ with Ark 99 $10^{4.2}$; Lane 7 =Negative control (PBS).

896 bp. However, strains Conn, Fla, JMK and Se-17 yielded one or two faint non-specific PCR products. After the annealing temperature was increased to 63°C, there were no non-specific bands observed for Conn, Fla, JMK and Se-17, whereas all four mass serotypes yielded strong 1026 bp product and all three Ark serotypes yielded 896 bp product. The result was reproducible. Therefore, the selection of primers and the optimization of the PCR condition are of great importance in the development of the multiplex PCR technique.

Mixed infections of different serotypes of IBV may occur due to the extensive use of multiple live vaccines. In order to test the feasibility of the technique in the diagnosis of Mass and Ark mixed infections, three individual combinations of different Mass and Ark EID₅₀ virus titers were used to co-infect 11-day-old embryonated eggs. The extracted RNAs were subject to the method. Both Mass and Ark were detected in all three co-infections. This indicated that the method can detect mixed infections of these two serotypes *in vivo* simultaneously.

Since Mass and Ark are among the common serotypes of IBV in the United States,^{25,29} the multiplex PCR developed in this study will be valuable for rapid identification, differential diagnosis and epidemiological studies of these two serotypes. Based on the results of this study, the development of multiplex PCR for other serotypes of IBV is possible which dramatically shortens the diagnosis process, especially when mixed infections of IBV serotypes are involved in outbreaks. Currently, the development of multiplex PCR for the other serotypes of IBV is in progress.

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REFERENCES

- Cavanagh, D. (1995). The coronavirus surface glycoprotein. In *The Coronaviridae*. (Siddell, S. G., ed.) pp. 90–3. New York, USA: Plenum Press.
- Cavanagh, D. & Naqi, S. A. (1997). Infectious bronchitis. In *Diseases of Poultry, 10th Edition*. (Calnek, B. W., ed.) pp. 511–26. Ames, Iowa, USA: Iowa State University Press.
- Cook, J. K. A. & Huggins, M. B. (1986). Newly isolated serotypes of infectious bronchitis virus: their role in disease. *Avian Pathology* **15**, 129–38.
- Gelb, J. Jr., Wolff, J. B. & Moron, C. A. (1991). Variant serotypes of IBV isolated from commercial layers and broiler chickens. *Avian Diseases* **35**, 82–7.
- Gelb, J. Jr. (1989). Infectious bronchitis. In *A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 3rd Edition*. (Purchase, H. G., Arp, C. H. & Pearson, J. E., eds). American Association Avian Pathologists.
- Gelb, J. Jr. & Killian, S. L. (1987). Serum antibody responses of chickens following sequential inoculations with infectious bronchitis virus serotypes. *Avian Diseases* **31**, 513–22.
- Karaca, K., Naqi, S. & Gelb, J. Jr. (1992). Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. *Avian Diseases* **36**, 903–15.
- Anderson, J. R., Jackwood, M. W. & Hilt, D. A. (1991). Polymerase chain reaction amplification of the genome of infectious bronchitis virus. *Avian Diseases* **35**, 216–20.
- Kwon, H. M., Jackwood, M. W. & Gelb, J. Jr. (1993). Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. *Avian Disease* **37**, 194–202.
- Lin, Z., Kato, A., Kudou, Y. & Ueda, S. (1991). A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction fragment length polymorphism. *Archive of Virology* **116**, 19–31.
- Keeler, C. L. Jr., Reed, K. L., Nix, W. A. & Gelb, J. Jr. (1998). Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer(S-1) gene. *Avian Diseases* **42**, 275–84.
- Way, J. S., Josephson, K. L., Pillai, S. D., Abbaszadegan, M., Gerba, C. P. & Pepper, I. L. (1993). Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction. *Applied and Environmental Microbiology* **59**, 1473–9.
- Lawrence, L. M. & Gilmour, A. (1994). Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their confirmation by multiplex PCR. *Applied and Environmental Microbiology* **60**, 4600–4.
- Kulski, J. K., Khinsoe, C., Pryce, T. & Christiansen, K. (1995). Use of multiplex PCR to detect and identify *Mycobacterium avium* and *M. intercellulare* in blood culture fluids of AIDS patients. *Journal of Clinical Microbiology* **33**, 668–74.
- Wang, H., Fadl, A. A. & Khan, M. I. (1997). Multiplex PCR for avian pathogenic mycoplasmas. *Molecular and Cellular Probes* **11**, 211–16.
- Karlsen, F., Kalantari, M., Jenkins, A., Petersen, E., Kristensen, G., Holm, R., Johansson, B. & Hagmar, B. (1996). Use of multiplex PCR primer sets for optimal detection of human papillomavirus. *Journal of Clinical Microbiology* **34**, 2095–100.
- Van der Heide, L., Kalbac, M., Brustolon, M. & Lawson, M. G. (1980). Pathogenicity for chickens of a reovirus isolated from turkeys. *Avian Disease* **24**, 989–97.
- Frey, M. L., Hanson, R. P. & Anderson, D. P. (1968). A medium for the isolation of avian mycoplasmas. *American Journal of Veterinary Research* **29**, 2163–71.
- Chomezynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytic Biochemistry* **162**, 156–9.

20. Niester, H. G., Lenstra, J. A., Spaan, W. J. *et al.* (1986). The peplomer protein sequence of the M41 strain of coronavirus IBV and its comparison with Beaudette strains. *Virus Research* **5**, 253–63.
21. Wang, L., Junker, D. & Collisson, E. W. (1993). Evidence of natural recombination within the S1 gene of infectious bronchitis virus. *Virology* **192**, 710–16.
22. Kwon, H. M. & Jackwood, M. W. (1995). Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. *Virus Genes* **9**, 219–29.
23. Wang, L., Junker, D., Hock, L., Ebiary, E. & Collisson, E. W. (1994). Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. *Virus Research* **34**, 327–38.
24. Binns, M. M., Boursnell, M. E., Tomley, F. M. & Brown, D. K. (1986). Comparison of the spike precursor sequences of coronavirus IBV strains M41 and 6/82 with that of IBV Beaudette. *Journal of General Virology* **67**, 2825–31.
25. Wang, L., Yuan, X. & Collisson, E. W. (1997). Experimental confirmation of recombination upstream of S1 hypervariable region of IBV. *Virus Research* **49**, 139–45.
26. Altschul, S. F., Gish, W., Miller, E. & Lipman, D. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–10.
27. Reed, L. J. & Muench, H. (1938). A simple method for estimating fifty percent end points. *American Journal of Hygiene* **27**, 493–7.
28. Kwok, S., Kellogg, D. E., Mckinney, N., Spasic, D., Goda, L., Levenson, C. & Sninsky, J. J. (1990). Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. *Nucleic Acid Research* **18**, 999–1005.
29. Warren, W. M. & Snyder, D. B. (1991). Epidemiology of infectious bronchitis in the United States. In *International Symposium on Infectious Bronchitis*. Pp. 53–7. Rauishholzhausen, Germany.