Comparison of a PCR-based diagnostic assay for *Mycoplasma pulmonis* with traditional detection techniques

S. Sanchez, K. Tyler, N. Rozengurt & J. Lida

Department of Veterinary Pathology, Royal Veterinary College, Royal College Street, London NW1 0TU, UK

Summary

Current diagnosis of infection by *Mycoplasma pulmonis*, an important pathogen of laboratory rodent colonies worlwide, is based on serological, histopathological and culture techniques which can be slow and unreliable. A polymerase chain reaction (PCR) assay for *M. pulmonis* diagnosis was compared to current diagnostic methods. This PCR based technique allows a more specific, sensitive and rapid diagnosis of *M. pulmonis* from various tissues by comparison with culture and histopathology.

Keywords Polymerase chain reaction; *Mycoplasma pulmonis*; validation; diagnosis

Murine mycoplasmosis, caused by *Mycoplasma pulmonis*, is one of the most important diseases of laboratory rats and mice. Because of its chronicity and slow cumulative mortality, *M. pulmonis* has an enormous impact on many parameters in rodent studies (Lindsey *et al.* 1971, Lindsey *et al.* 1978, Cassell *et al.* 1986); moreover, infection of the urogenital mucosa can diminish breeding efficiency (Cassell *et al.* 1986).

The most effective current method of control is the establishment and maintenance of *M. pulmonis*-free breeding colonies. This requires the careful screening of breeding stocks using rigorous detection methods and strict maintenance of the physical barriers. Caesarean rederivation is used to eliminate infections once they are established in the colony, rigorous testing of offspring is essential because of the possibility of foetal contamination or transplacental transmission (Cassell *et al.* 1986).

Correspondence to: Dr S Sanchez

The detection of *M. pulmonis* infection requires the use of one or more of the following procedures: a) cultural isolation; b) demonstration of mycoplasmas *in situ* by labelled specific antibodies (immunofluorescence, immunoperoxidase); c) histopathology, and d) serological methods (Cassell *et al.* 1986).

a) Isolation and cultivation of mycoplasmas can be laborious and time consuming, it necessitates specialized culture techniques and expensive culture media (Cassell *et al.* 1979).

b) The demonstration of mycoplasmas in infected tissue is also laborious and time consuming.

c) Morphological diagnosis made on the basis of microscopic evaluation of the respiratory and genital tracts, can only be presumptive as microscopic changes are minimal or absent during the first weeks of a mild disease process.

d) Conventional serology and tests such as complement fixation and haemagglutination inhibition, which rely on functional assays, are of limited value because serum antibody levels of *M. pulmonis* are often low, and

Accepted 19 August 1993

they appear as late as the third month after exposure to the agent (Cassell *et al.* 1986). Thus early infection and spread cannot be detected serologically without a risk of false negative results. Enzyme-linked immunosorbent assay (ELISA) (Horowitz & Cassell 1978) is quicker and more sensitive, but can often give false positive results due to cross reaction with *M. arthritidis* in some tests. False positive and false negative ELISA test results can be related to reactive substances within the specimen such as lysozyme, antinuclear antibodies, protease and bacterial products (Bullock & Walls 1977, Essink *et al.* 1985, Naot *et al.* 1981, Yolken 1985).

DNA and RNA probes have proved sensitive and specific diagnostic tools for numerous infectious agents including *Mycoplasma* species (Hyman *et al.* 1987, Khan *et al.* 1987, Razin *et al.* 1987, Santha *et al.* 1987). In general though, these protocols are too lengthy and costly for routine diagnosis.

PCR, in contrast, requires minimal sample preparation, which can be performed routinely on multiple samples. Over a course of a few hours, PCR can be as specific and more sensitive than other established molecular biological techniques, and is extremely economic when used routinely as a diagnostic tool. In this paper we have sought to compare the performance of PCR as a diagnostic technique with existing routine diagnostic methods in the detection of natural *M. pulmonis* infection in rat colonies.

Materials and methods

Mycoplasma strains and species

Eleven different strains of *M. pulmonis* isolated from unusual sites and rodent species (received from Dr A. Hill including 2 of the reference strains: ASH, NIH reference strain), and 12 other *Mycoplasma* species (*M. salivarium*, *M. pneumoniae*, *M. orale*, *M. neurolyticum*, *M. hyorhinis*, *M. hominis*, *M. arginini*, *M. caviae*, *M. cavipharyngis*, *M. collis*, *M. cricetulae*, *A. laidlawii* received from Dr A. Hill), were cultured on PPLO broth and agar plates, both supplemented with Mycoplasma supplement G (Oxoid, Ltd), phenol red (0.1% w/v), the media of those mycoplasmas that ferment Glucose were supplemented with 1% final concentration of Glucose and the media of those mycoplasmas that utilize Arginine were supplemented with 1% final concentration of Arginine (Cassell & Hill 1979) aerobically (except for the primate species that were cultured anaerobically) at 37°C. All cultures on solid media were kept in a humid atmosphere. Once mycoplasma growth was demonstrated on the plates the broth was boiled for 10 min, centrifuged for 3 min at $10\,000 \times g$, and the supernatant was used as a template for PCR.

Bacteria species

The bacteria *Haemophilus pleuropneumoniae*, *Proteus mirabilis*, *Pasteurella pneumotropica*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were cultured overnight in 2% horse serum enriched broth.

Purification of DNA from mycoplasma and bacteria

Both the bacteria and *M. pulmonis* were cultured in liquid media.

The mycoplasma culture was spun down at $10\,000 \times g$ for 15 min at 4°C. The pellet was resuspended in 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0), 1% SDS. Following 2 freeze/thaw cycles the mixture was incubated with RNAase (50 μg/ml) at 37°C for 1 h. DNA was extracted with phenol and chloroform, precipitated with 2.5 volumes of ethanol, washed with 70% ethanol, dried under vacuum and dissolved in 100 μl of distilled water.

The bacteria cultures were processed as follows, spun down at $10\,000 \times g$ for 15 min at 4°C. The pellet was resuspended in 1.5 ml solution 1 (1 ml 1 M EDTA pH 8.0, 1.5 ml 1M NaCl, 7.5 ml water, 10 mg Lysozyme); incubated for 15 min at 37°C, and frozen at −70°C. 12.5 ml of solution 2 (0.4 ml 5 M NaCl, 2 ml 1 M Tris-HCl, 0.2 SDS, 20 ml water) was added, and the mixture thawed at 65°C. After a second

freeze/thaw, the mixture was chilled on ice and the DNA was extracted with phenol and chloroform, precipitated with ethanol, dried under vacuum and dissolved in TE (10 mM Tris-HCl 1 mM EDTA) pH 8.0 with 50 μg/ml RNAase. After incubating for 1 h at 37°C, DNA was re-precipitated, dried under vacuum and dissolved in 100 μl TE.

Tissues studied

Thirty cases of suspected mycoplasmosis in rats were analysed from 2 different sources. Both were typical examples of university animal houses, where the rooms were on a 12:12 h light/dark cycle, and temperature was regulated at 21–24°C but without regulated humidity. Food and water was provided *ad libitum*.

From source I, 8 SD albino rats, 5 females and 3 males kept under non-barrier conditions were chosen at random from one room.

From source II, 22 MR albino rats, 13 females and 9 males, kept under non-barrier conditions were chosen at random from one room.

The genetic and microbiological status of both colonies was unknown. Clinical signs of respiratory disease were present in the rats. Ages varied between 2 months and one year. The tissues selected for study were lungs, uterus and urethra. Germ-free SD rats were used to generate tissues to be used as negative controls.

Treatment of tissue samples for PCR amplification

Tissues collected from euthanazed animals were frozen immediately in liquid nitrogen, and kept for a later processing or processed immediately as follows: Approximately 1 g of each of the frozen tissues was ground into a fine powder, placed in a sterile glass container with 1 ml of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0), 1% SDS, boiled for 10 min, spun at 10 000 × *g* for 3 min and the supernatant used as template for the PCR.

Preparation of oligonucleotides and labelled probes

The oligonucleotide primers were synthesized according to the consensus nucleotide sequence of a 2.3 kilobase pair (kbp) fragment previously described (Kunita *et al.* 1989) which is specific for *M. pulmonis*. One set of 20 base oligonucleotide primers was synthesized according to the published sequence (Kunita *et al.* 1990). The length of the genome fragment targeted was 245 bp. A central probe of 30 bp was synthesized and radio-labelled with alpha 32P-dCTP (Amersham), by the random primer method (Feinberg & Vogelstein 1983).

Polymerase chain reaction

The target sequence was amplified in a total reaction volume of 100 μl, consisting of 50 mM KCl. 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 1 μM each of the primers, 200 μM each of the deoxynucleotide triphosphates (dATP, dGTP, dTTP and dCTP) and 5 μl of the sample as a template. Then 2.5 units of Taq polymerase (Perkin Elmer, Cetus) were added to the reaction mixture which was then overlaid with 50 μl of mineral oil. Samples were amplified using a thermocycle of: 30 sec at 95°C, 30 sec at 50°C and 90 sec at 70°C, for 30 cycles. The final DNA extension was continued for 10 min. All clinical samples were subjected to a second round of the amplification using the same parameters as above in order to maximize sensitivity.

Analysis of amplified products

Ten μl of the reaction product was loaded onto a composite agarose gel consisting of 3% NuSieve agarose and 1% non-modified agarose and ethidium bromide (0.5 μg/ml) and run in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) for 45 min at 100 V. The gel was photographed using a UV transilluminator to detect the amplified DNA.

For Southern hybridization, agarose gel-resolved samples were transferred to nylon membranes by capillary blotting, and fixed by transillumination with UV light (240 nm) for 30 sec.

Filters were pre-hybridized for 1 h at 65°C in a solution of 5 × Denhardt's

solution, (4×) standard saline citrate (SSC), and 0.5% SDS before adding the labelled probes with 100 mg/ml denatured salmon sperm DNA. Hybridization took place at 65°C overnight. Post-hybridization washes consisted of 1 rinse in 1×SSC and 0.1% SDS, followed by rinses in 0.2×SSC and 0.1% SDS. Autoradiography was performed at −70°C for 12–24 h using an intensifying screen and Kodak X-AR film.

Serological testing

ELISA was used for the detection of antibodies against *M. pulmonis* (the modified procedure of Horowitz & Cassell 1978). Rabbit antibody against heavy and light chains of rat IgG was conjugated to peroxidase. Absorbency values were determined at 492 nm using an automatic 96 well spectrophotometer (Titertrek. Multiskan Plus MKII). A base line absorbency value of 0.100 was previously determined by assaying negative rat sera. Following calibration assays, only absorbency values equal or greater than 0.200 were considered positive, whereas absorbency values between 0.100 and 0.200 absorbency units were considered borderline.

Isolation and histopathology

Swabs from the nasopharynx and urogenital tract were deposited in PPLO broth and cultured at 37°C (Cassell *et al.* 1979). Cultures were kept for a minimum of 21 days before assessment (reported negative). Suspected mycoplasma colonies were identified by growth inhibition test (Standbridge *et al.* 1967).

Part of the lungs were collected and fixed with 10% neutral buffered formalin. After fixation, all tissues were embedded in paraffin and sectioned. Sections were stained with haematoxylin and eosin and examined for lesions compatible with those of murine mycoplasmosis. Lesions were graded as follows: (0) no detachable lesions; (+1) congestion, mild perivascular cuffin or lymphoid hyperplasia: less than 25% of total lung area affected; (+2) moderate lymphoid hyperplasia, perivascular cuffin: (25–50% of total area affected); (+3) massive lymphoid hyperplasia, consolidation and bronchiectasis (greater than 50% of total lung area affected).

Results

PCR amplification assay

PCR amplification using 11 isolates of *M. pulmonis* in the form of mycoplasmal DNA or boiled extract as template, yielded a DNA fragment of 245 bp, which when sequenced was 100% homologous with the predicted sequence (Kunita *et al.* 1990) (Fig 1). All samples were positive but vary in intensity presumably due to varying amounts of sample DNA in each specimen. This is to be expected, since the rapid/boiling protocol for sample preparation does not quantitate the initial amount of DNA and samples were drawn from widely divergent sources. In this case the test merely says yes or no. However, when rodent genomic DNAs, other *Mycoplasma* species or bacterial DNA were used as templates no amplified products were detected. Using Southern blotting, *M. pulmonis* specific DNA probes hybridized to the PCR amplified 245 bp fragments (Fig 2). Restriction digestion of the amplified fragment with Hpa I (data not shown) yielded two fragments of 160 and 185 bp as predicted (Kunita *et al.* 1990).

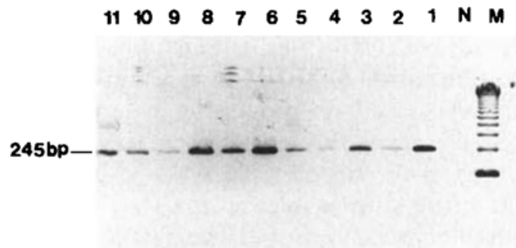

Fig 1 Specificity of PCR amplification. An amplified 245 bp fragment was detected when each of 11 different Mp strains was subjected to PCR amplification and analysis by agarose gel electrophoresis. Marker (M) is 123 bp concatameric ladder. Negative control (N) is boiled extract of *E. coli*

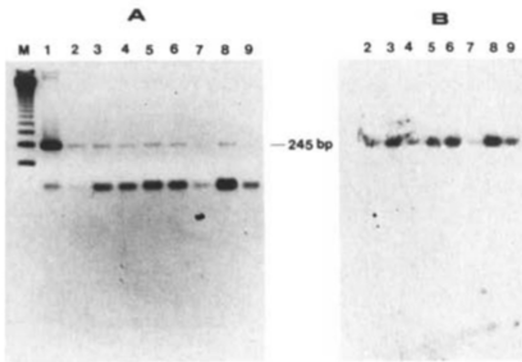

Fig 2 Validation of an amplified 245 bp fragment from 8 positive tissue samples. A: Analysis by agarose gel electrophoresis—Marker is a 123 bp concatameric ladder, lane 1 is the positive control. Lanes 2–8 positive tissue samples. B: Autoradiograph of southern blot from same agarose gel. The validatory probe used was 30 bases long and synthesized to a predicted sequence from the centre of the fragment

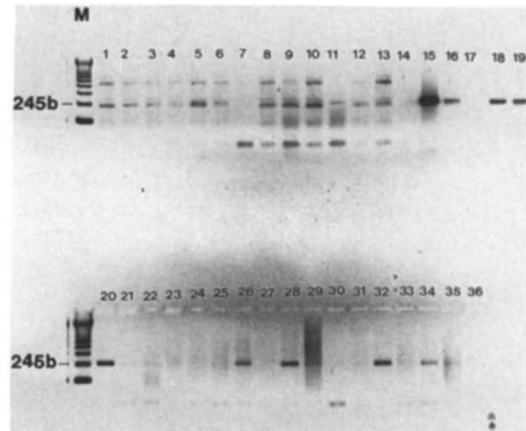

Fig 3 A run of tissue samples following the first round of PCR amplification and agarose gel electrophoresis. Here a preliminary yes/no answer is obtained for each of 34 samples according to the presence or absence of the diagnostic 245 bp band for *M. pulmonis*. Includes positive and negative controls, numbers 20 and 36 of bottom line. Lung samples are presented first sequentially numbers 1 to 19 and then the uterus samples numbers 21 to 35. As can be clearly seen all samples are positive except for numbers 7, 14, 17, 21, 22, 23, 24, 25, 27, 30, 31 and 33. All these negative samples were submitted to a second round of amplification after which all lung samples were positive, uterus samples remained negative. Other bands sometimes occur but can be considered irrelevant to the assay

Multiple banding was occasionally observed in some *M. pulmonis* samples on amplification, but in all positive cases a band of the expected 245 bp was observed (Fig 3). Slight differences in band migration are caused by imperfections in the gel as well as inconsistencies in the amount of DNA loaded. The diagnostic band is reproducibly identical in all cases and always hybridizes to the central 30 bp probe if blotted.

PCR sensitivity assay

The sensitivity of the PCR assay was measured by consecutive dilution of purified *M. pulmonis* DNA, the amplification procedure used was identical to that used for the clinical samples. As little as 1 pg of *M. pulmonis* DNA can be detected by this assay in contrast to the much larger amounts required for detection by existing methods (Fig 4).

Comparison of methods

Thirty *M. pulmonis* isolates from 2 groups of clinically affected rats were used to compare the PCR diagnostic assay with existing methods. Rats Nos 1 to 8 came from source I and had been subjected to treatment with 1 g of Tylan Soluble powder (Elanco Products Ltd) per litre of drinking water during 10 days, repeating every month for 3 months prior to sampling. Animals Nos 9 to 30 came from source II and had been subjected to treatment with 800 mg of oxytetracycline per litre of drinking water for 5 days per month during 3 months before sampling. All these treatments were prescribed by Named Veterinary Surgeons of the respective animal houses as a way of improving the welfare of the rat colonies. All these rats were positive by PCR assay.

Macroscopic examination

Necropsy examination revealed circumscribed purple lesions in the lungs of varying severity but no other abnormalities.

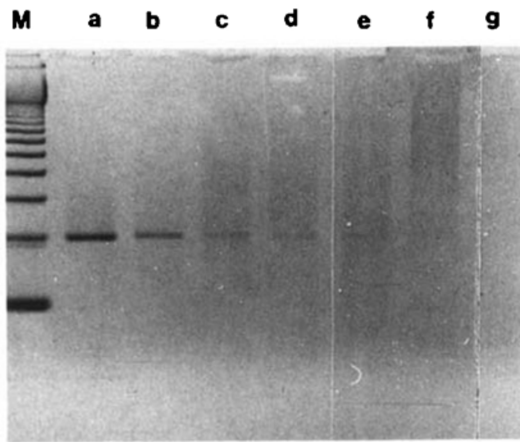

Fig 4 Sensitivity assay by serial dilution of Mp genomic DNA. Subsequent to extraction DNA was diluted in factors of 10 from an initial concentration of 10 mg/ml to 1 pg/ml. M 123 bp concatameric marker. 1) 100 μ/ml, 2) 10 μg/ml, 3) 1 μg/ml, 4) 100 ng/ml, 5) 10 ng/ml, 6) 1 ng/ml 7) 100 pg/ml. (5 μl of sample was used in each reaction hence the limit of sensitivity was observed to be 5/1000 * 100 pg = < 1 pg)

Culture and histopathology examination

Six rats, out of 30, were culture-positive, but only 2 of the isolates from the conventional nasopharynx swabbing site were successfully cultured. All other rats (females) isolates were obtained from the urogenital tract (Table 1). Histopathology examination revealed lesions in the lung samples Nos 1 to 8 (from source I) but only 2 (25%) lesions were indicative of mild mycoplasmosis the other 6 had their lungs consolidated due to interstitial pneumonia with thickening of the alveolar walls and some lymphocytic infiltrates in the proximity of blood vessels. Most of these lesions were suggestive of viral respiratory infections such as Sendai virus or pneumonia virus of mice (PVM). In rats from source II, 4 (18.18%) of the animals studied had severe to moderate lesions, 9 (40.90%) showed mild lesions, and 9 (40.90%) did not show any lesion that resembled chronic murine mycoplasmosis.

Serology

All of the 30 rats used in the study were ELISA positive with high titres. Fifteen had lung lesions ranging from mild to severe, but none of these were culture positive.

Table 1 A comparison of common techniques and PCR in the diagnosis of *Mycoplasma pulmonis*. Results obtained from routine diagnosis, on analysis of 30 tissues by 4 different methodologies

Tissue sample number	Histology	Serology	Culture	PCR
1	0	+	−	+l
2	0	+	−	+l
3	0	+	+u	+u
4	0	+	−	+l
5	+1	+	−	+u
6	0	+	−	+l
7	+1	+	−	+l
8	0	+	+u	+ul
9	+1	+	−	+l
10	0	+	+u	+ul
11	0	+	+u	+ul
12	0	+	+l	+ul
13	0	+	−	+l
14	+3	+	−	+l
15	+1	+	−	+l
16	0	+	−	+l
17	0	+	−	+l
18	0	+	−	+l
19	0	+	−	+l
20	+1	+	−	+l
21	+1	+	−	+l
22	0	+	−	+l
23	0	+	−	+l
24	+2	+	−	+l
25	+3	+	−	+l
26	+2	+	−	+l
27	+1	+	−	+l
28	+1	+	−	+l
29	+1	+	−	+l
30	+1	+	+l	+l

+ = positive; − = negative; 0 = no pathology suggestive of mycoplasma; +1 = mild lesions, <25% of lung; +2 = moderate lesions, 25–50% of lung; +3 = massive lesions >50% of lung; u = urogenital tract; l = lung

Discussion

PCR has already been applied successfully to the detection and confirmation of a wide variety of infections caused by viruses, parasites and bacteria including *M. pulmonis* (Kunita *et al.* 1990, Harasana *et al.* 1990, Schriefer *et al.* 1991, Olive 1989), but a comparison between it and existing methods for efficiency as a diagnostic tool

has been overdue. This study has shown the sensitivity of assay by PCR, being able to detect as little as 1 pg (<5 Mp genomes) in 5 μl of sample supernatant. PCR assay's specificity is such that it will pick its target sequence out of a massive background of non *M. pulmonis* genomic DNA concentrated from a million cells and amplify it to readily detectable levels.

By screening a wide range of possible DNA background controls it was shown that the primers used were sufficiently specific to give positive results with all available *M. pulmonis* strains, yet remain negative with other mycoplasma species and bacterial flora of the nasopharyngeal tract.

Although in this study serology and PCR yielded equivalent results (Table 1), serology can often be misleading suffering from the following disadvantages:

False serological 'positives' that may occur following recovery in antibiotic-treated positive animals.

Antibody titres rising only 3 to 4 months after infection, leaving a gap during which false negatives may occur (Cassell *et al.* 1986).

Cross-reactivity of antibodies to haptenic groups, between both host and medically distinct though serologically similar pathogens.

The serological heterogeneity of *M. pulmonis*, significantly undermining the reliability of serodiagnosis.

Different strains of mice differ in humoral immune responses, with up to 12-fold difference in IgG production (Simecka *et al.* 1987, Davidson *et al.* 1988) leading to wide inter-sample variation which prevents a clear cut-off between positives and negatives. PCR detects only *M. pulmonis* DNA and since non-viable mycoplasmas are degraded and cleared very rapidly from mucosal tissue, only viable and, therefore, current infection is detected.

The limitations of other common techniques were demonstrated in comparison with PCR. Histopathological lesions associated with murine respiratory mycoplasmosis, are influenced by the genetic background, age, environmental factors (Simecka *et al.* 1987) and the strain of *M. pulmonis* (Davidson *et al.* 1988). They are thus rendered less reliable for diagnosis, but were nonetheless better than straight culture assays, especially in the group 9–30. The low isolation rate of this study may well be related to the limitations of the culture technique used. Culture success relies heavily in the composition of the media used. Mycoplasmicidal substances do exist in many culture samples. The addition of substances such as reinechate and lysophospholypase in the media may neutralize growth inhibitors as do the dilutions of the samples in mycoplasmal broth or both (Tully & Rusk-Nielsen 1967, Mazda *et al.* 1973), thus increasing the isolation rate. Also, mycoplasmas are known to become host adapted in chronic infections and they are no longer culturable (Decker & Barden 1975).

Whilst a degree of specificity similar to that obtained with PCR can be achieved using techniques such as blotting or *in situ* hybridization, PCR is several factors more sensitive (Kunita *et al.* 1989, Harasana *et al.* 1990) and quicker.

This study was conducted on rats which had already received some therapeutic treatment. Unlike serology, PCR can demonstrate the point at which treatment is successful and all *M. pulmonis* is eliminated. In comparison, serology would continue to generate positive results for several months thereafter. Although in this study tissue samples were taken from killed rats, PCR enables mucosal swabs to be used (Sanchez *et al.* unpublished) abrogating the need to kill valuable animals.

Acknowledgements The authors wish to acknowledge the valuable assistance of Dr N. Taich and Mr A. Sebesteny; to thank Imperial Chemical Industries Plc for financial assistance towards this project and to commend Mrs K. Lockey for the typing of the manuscript.

References

Bullock SL, Walls KW (1977) Evaluation of some of the parameters of the Enzyme-Linked Immunosorbent Assay. *Journal of Infectious Diseases* **136** (suppl) 279–85

Cassell GH, Davis JK, Simeka JW, Lindsey JR, Cox NR, Ross S, Fallon M (1986) Mycoplasmal infections: Disease pathogenesis implications for biomedical research, and control. In: *Viral and mycoplasmal infections of laboratory rodents* (Bhatt PN, Jacoby RO, Morse III HC, New AE, eds). New York: Academic Press Inc, pp 87-130

Cassell GH, Hill A (1979) Murine and other small animal mycoplasmas. In: *The Mycoplasmas Vol II* (Tulley JG, Whitcomb RF, eds). New York: Academic Press Inc, pp 235-73

Davidson MK, Lindsey JR, Parker RF, Tully JG, Cassell GH (1988) Differences in virulence for mice among strains of *Mycoplasma pulmonis*. *Infection and Immunity* **56**, 2156-62

Decker JL, Barden JA (1975) *Mycoplasma hyorhinis* arthritis of swine: A model for rheumatoid arthritis? In: *Immunological aspects of rheumatoid arthritidis. Rheumatology. Vol. 6* (Clot J, Sany J, eds). Basel-Karger pp 338-45

Essink AWG, Arkesteijn GJ (1985) Interference of lysozyme in the Sandwich Enzyme-Linked Immunosorbent Assay (ELISA). *Journal of Immunology Methods* **80**: 91-6

Feinberg AP, Volgelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Annals of Biochemistry* **132**: 6-13

Harasana R, Koshimizu K, Vemori T, Takeda O, Asada K, Kato I (1990) The polymerase chain reaction for *Mycoplasma pulmonis*. *Microbiology Immunology* **34**: 393-5

Horowitz SA, Cassell GH (1978) Detection of *Mycoplasma pulmonis* by an enzyme immunosorbent assay. *Infection and Immunity* **22**: 161-70

Hyman HC, Yogev D, Razin S (1987) DNA probes for the detection and identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *Journal of Clinical Microbiology* **25**: 726-8

Khan MI, Kirkpatrick BC, Yamamoto R (1987) A *Mycoplasma gallisepticum* strain-specific DNA probe. *Avian Diseases* **31**: 907-9

Kunita S, Terada E, Ghoda A, *et al.* (1989) A DNA probe for specific detection of *Mycoplasma pulmonis*. *Experimental Animals* **38**, 215-19

Kunita S, Terada E, Goto K, Kagiyama N (1990) Sensitive detection of *Mycoplasma pulmonis* by using the polymerase chain reaction. *Experimental Animals* **39**, 104-7

Lindsey JR, Baker HJ, Overcash RG, Cassell GH, Hunt CE (1971) Murine chronic respiratory disease. Significance as a research complication and experimental production with *Mycoplasma pulmonis*. *American Journal of Pathology* **64**: 675-716

Lindsey JR, Cassell GH, Baker HJ (1978) Diseases due to mycoplasmas and rickettsias. In: *Pathology of Laboratory Animals Vol III* (Bernishke K, Garner FM, Jones TC, eds). New York: Springer-Verlag, pp 1481-550

Mazda PA, Taylor-Robinson D (1973) New approaches to the isolation of Mycoplasmas. *Medical Microbiological Immunology* **158**, 259-60

Naot Y, Barnett EV, Remington JS (1981) Method for avoiding false positive results occurring in immunoglobulin M Enzyme Linked Immunosorbent Assays due to presence of both Rheumatoid factor and antinuclear antibodies. *Journal of Clinical Microbiology* **14**, 73-8

Olive DM (1989) Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with thermostable DNA polymerase. *Journal of Clinical Microbiology* **158**, 1154-7

Razin S, Hyman HC, Nur I, Yogev D (1987) DNA probes for the detection and identification of mycoplasmas (mollicutes). *Israel Journal of Medical Sciences* **23**, 735-41

Santha M, Brug K, Rasko I, Stipkovits L (1987) A species specific DNA probe for the detection of *Mycoplasma gallisepticum*. *Infection and Immunity* **55**, 2857-9

Schriefer ME, Sacci JB, Wirtz RA, Azad AF (1991) Detection of Polymerase Chain Reaction-Amplified malarial DNA in infected blood and individual mosquitoes. *Experimental Parasitology* **73**, 311-16

Simecka JW, Davis JK, Cassell GH (1987) Specific versus nonspecific immune response in murine respiratory mycoplasmosis. *Israel Journal of Medical Sciences* **23**, 485-9

Stanbridge E, Hatflick L (1967) Growth inhibition test for identification of *Mycoplasma* species utilizing dried anti-serum impregnated discs. *Journal of Bacteriology* **93**, 1392-6

Tully JG, Rusk-Nielsen R (1967) Mycoplasmas in leukaemic and non-leukaemic mice. *Annals of the New York Academy of Sciences* **443**, 345-52

Yolken RH (1985) Solid phase Enzyme Immunosorbent Assays for the detection of microbial antigens in body fluids. In: *Manual of Clinical Microbiology. 4th Edn.* (Lenette EH, Balows A, Hausler WJ Jr *et al.* eds). Washington DC: American Society of Microbiology, pp 949-58