Etiological investigation of multiple respiratory infections in cats

B. Di Martino, C.E. Di Francesco, I. Meridiani, F. Marsilio

Department of Comparative Biomedical Sciences, University of Teramo, Italy

SUMMARY

In order to evaluate the relevance of multiple infections in domestic cats with Upper Respiratory Tract Disease (URTD) one hundred animals with clinical signs were investigated for detection of Feline Herpesvirus type-1 (FHV-1), *Chlamydophila felis*, Feline Calicivirus (FCV) and *Bordetella bronchiseptica* from mucosal swabs.
Forty-seven cats were positive for FCV, 42 cats for FHV-1, 26 for *B. bronchiseptica* and 8 for *C. felis*. Dual or multiple infections were found in 33 of examined animals.
Our results document that FCV and FHV-1 are the major recognized cause of URTD, although infections associated with other pathogens such as *B. bronchiseptica* or *C. felis* are also common in cats.

KEY WORDS: Cat, Upper respiratory tract disease, Mucosal swabs, Diagnosis

Received March 08, 2007 *Accepted* May 14, 2007

INTRODUCTION

Upper Respiratory Tract Disease (URTD) is a common infection in domestic cats. Several studies reported that Feline Calicivirus (FCV), Feline Herpesvirus type-1 (FHV-1) and *Chlamydophila felis* are the major causative agents (Binns *et al.,* 2000; Sykes *et al.* 2001; Cay *et al.,* 2002; Helps *et al.,* 2003; Bannasch and Foley 2005; Holst *et al.,* 2005). However, the related data are not always comparable since the presence of each pathogen may differ according to feline population features, anatomic site of sampling and laboratory assays used for the diagnosis.
In FCV and FHV-1 infections the severity of clinical signs may depend on viral strain, animal age and secondary infections. Common signs are nasal and ocular discharge, sneezing, dyspnea and coughing. In addition, oral ulcerations and chronic gingivitis are observed in cats with FCV infection and abortion may occur in pregnant cats infected with FHV-1 (Sykes 2001). *C. felis* causes primarily ocular lesions characterized by acute or chronic conjunctivitis and blepharospasm associated with serous or mucopurulent ocular discharge (Sykes 2005).
Recently, *Bordetella bronchiseptica* was recognized as a primary pathogen in URTD-affected cats (Binns *et al.,* 1999; Pennisi *et al.,* 1999; Pasmans *et al.,* 2001, Helps *et al.,* 2005). In experimental infections *B. bronchiseptica* can determine a clinical onset ranging from mild respiratory signs to lethal pneumonia particularly in kittens (Jacobs *et al.*, 1993, Coutts *et al.*, 1996; Welsh *et al.*, 1996; Hoskins *et al.*, 1998).
In URTD-affected cats, each pathogen can be found alone or associated in dual or multiple infections. Dual or multiple infections with FCV, FHV-1 and *C. felis* have been reported (Mochizuki *et al.,* 2000; Sykes *et al.,* 2001; Cai *et al.,* 2002; Helps *et al.,* 2003; Dawson *et al.,* 2004). Moreover, few data relating co-infections with *B. bron-*

Corresponding author
C.E. Di Francesco
Department of Comparative Biomedical Sciences
University of Teramo,
Piazza Aldo Moro, 45
64100 Teramo, Italy
E-mail: cedifrancesco@unite.it

chiseptica and its effective role in URTD are available (Binns *et al.,* 2000; Helps *et al.,* 2005).
In Italy, the studies carried out up to now are incomplete and pointed out the important role of FHV-1 and *C. felis* as joint agents of ocular and respiratory diseases in cats (Di Francesco *et al.,* 2001; Marsilio *et al.,* 2004), but there are no data on multiple infections with FCV and/or *B. bronchiseptica*. Recently, Polymerase Chain Reaction (PCR) assays have been developed for detection of FCV and *B. bronchiseptica* from ocular and pharyngeal swabs collected from cats with respiratory syndrome (Di Martino *et al.,* 2005, Marsilio *et al.,* 2005). Compared with traditional isolation assay, these methods offer some advantages such as short execution time, high sensitivity and specificity, and rapid identification of pathogens that are difficult to isolate (Sykes, 2005). In this note we report the results of a study carried out on 100 cats with respiratory syndrome to detect FHV-1 and *C. felis* by a duplex-PCR and Restriction Fragment Length Polymorphism (RFLP) analysis, FCV by a nested PCR and *B. bronchiseptica* by a specific PCR, in order to evaluate the relevance of multiple infections in domestic cats with URTD.

MATERIALS AND METHODS

Clinical samples
Ninety-seven pharyngeal swabs and ninety-two conjunctival swabs were collected from 100 cats with URTD-related symptoms from October 2002 through February 2004. It was not possible to collect pharyngeal and conjunctival swabs from 3 and 8 cats, respectively. The samples were collected in private and public veterinary clinics in Isernia, Ascoli Piceno, Rome and Teramo areas (Central Italy). Each sample came with an anamnestic card reporting information on the clinical signs and the possible pharmacological or vaccinal treatments.
The samples were collected using suitable sterile swabs, dipped in Dulbecco's modified Eagle's medium (DMEM), kept at +4°C during the transfer to the laboratory and stored at -80°C until testing.

DNA and RNA extractions
Nucleic acid extraction from the 189 mucosal swabs were performed using a commercial kit (QIAamp UltraSens Virus kit, Qiagen, Germany), useful for simultaneous extraction of DNA and RNA from cell-free liquids. The protocol followed was that suggested by the manufacturer.

Duplex-PCR amplification for FHV-1 and Chlamydophila spp.
Duplex PCR was applied using the same primers sets reported previously (Marsilio *et al*. 2004) (Table 1). The target sequences are:
- 321-bp segment included in the TK gene of FHV-1 (GeneBank Accession Number M26660);
- 590-bp sequence included in the OMP2 gene encoding the Outer Membrane Protein of *Chlamydophila* spp. (GeneBank Accession Number U65942).

Briefly, the assay was performed in a single tube containing PCR buffer HotMaster Taq 1X, 2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 2,5 U of HotMaster Taq DNA Polymerase (Eppendorf, Germany), 50 pmol of FHV-F and FHV-R primers, 100 pmol of Chla-AF and Chla-AR primers; 4 µl of total DNA isolated was added to reaction mixture. The amplification was performed using the following conditions: 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min followed by a final extension of 7 min at 72°C.
The method was carried out on all mucosal swabs. The DNA extracts from a wild strain of FHV-1 and *C. felis* as positive controls and mock-infected CrFK as negative controls were included.

C. felis identification by Restriction Fragment Length Polymorphism analysis (RFLP)
PCR products resulting from amplification of the OMP2 gene of *Chlamydophila* spp. were collected and subjected to species identification by RFLP analysis with *Hind*III (Biolabs, New England). This enzyme is able to cut *C. felis* PCR product into two fragments, 122 bp and 468 bp long, thus discriminating *C. felis* from other species (Marsilio *et al.,* 2004). Digested fragments were analysed using 3% agarose gel electrophoresis and visualization with ethidium bromide staining and ultraviolet transillumination.

Nested PCR amplification for FCV
The target sequence of RT-PCR amplification is a 924-nucleotide region of the capsid gene (ORF2), equivalent to residues 5322 to 6246 of FCV strain

TABLE 1 - *Primer sets used for PCR assays.*

Primer	*Sequence 5' to 3'*	*Position*	*GeneBank accession number*	*Amplicon Size (bp)*
FHV-F FHV-R	TGTCCGCATTTACATAGATGG GGGGTGTTCCTCACATACAA	328-349 629-649	M26660	321
Chla-AF: Chla-AR:	ATGTCCAAACTCATCAGACGAG CCTTCTTTAAGAGGTTTTACCCA	961-982 1525-1548	U65942	590
Cali 1 Cali 2	AACCTGCGCTAACGTGCTTA CAGTGACAATACACCCAGAAG	5322-5341 6227-6247	M86379	924
Cali 3 Cali 4	TGGTGATGATGAATGGGCTC ACACCAGAGCCAGAGATAGA	5514-5534 5971-5991	M86379	467
Bbf Bbr	AAGGTCGTGCAACTGCCCAA ATGTGCTGGCCGTTGAGGT	157-176 422-441	AY017346	284

F9 (GenBank Accession Number M86379). Primers sets were described previously (Marsilio *et al.* 2005) (Table 1). Briefly, one step RT-PCR was performed in a total reaction volume of 50 µl containing PCR buffer HotMaster Taq 1X, 3.5 mM di $MgCl_2$, 2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 10 U of RNase inhibitor, 50 U of MuLV RT, 1.25 U of HotMaster Taq DNA Polymerase (Eppendorf, Germany) and 10 pmol of Cali 1 and Cali 2 primers; 5 µl of each extracted sample was added to the reaction mixture. Synthesis of cDNA was carried out at 42°C for 45 min, followed by a step at 94°C for 5 min to inactivate the MuLV RT. Then the target sequence was amplified at the following conditions: 35 cycles of 94°C for 1 min, 57°C for 45 s and 72°C for 1 min followed by a final extension of 7 min at 72°C. For the nested PCR, 1 µl of the RT-PCR product was subjected to a second reaction to amplify a 467-bp long fragment. The reaction was performed using internal primers Cali3 and Cali4 and the same conditions of the first step amplification. At the end of nested PCR, 8 µl of the reaction product were analysed by 2% agarose gel electrophoresis and visualization by UV transillumination. The method was carried out on 189 mucosal swabs, including an FCV F9 strain extract as positive control and mock-infected CrFK as negative control.

PCR amplification for B. bronchiseptica

The target sequence for PCR amplification is a 284 bp fragment of the fimbria (fim3) gene of *B. bronchiseptica* SB283 strain (Genbank Accession Number: AY017346). Selected primers are described in Table 1.

The amplification was done as previously described (Di Martino *et al.,* 2005). Briefly, the reaction was performed with 4 µl of extracted DNA, PCR buffer HotMaster Taq 1X, 3.5 mM of $MgCl_2$, 2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 1,25 U of HotMaster Taq DNA Polymerase (Eppendorf, Germany) and 10 pmol of Bbf and Bbr primers. Amplification was performed under the following conditions: DNA was firstly denatured at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 58°C for 45 s and 72°C for 1 min with an additional incubation of 5 min at 72°C to complete extension.

The method was carried out on 189 mucosal swabs, including a DNA extract from *Pseudomonas aeruginosa* as negative control and *B. bronchiseptica* Onselen strain DNA as positive control.

RESULTS

The results of the PCR assays for detection of FCV, *B. bronchiseptica*, FHV-1 and *C. felis* carried out on 92 conjuctival and 97 pharyngeal swabs collected from 100 cats with URTD are summarized in Table 2.

The duplex PCR for the diagnosis of FHV-1 and *Chlamydophila* spp. produced two amplicons of 321 and 590 bp, respectively. The nested PCR for

TABLE 2 - *PCR results: single, dual and multiple infections in cats affected by URTD.*

Single infections	*Dual infections*	*Multiple infections*	*Total for each pathogen*
FCV 17	FCV/FHV 13	FHV/FCV/*B. bronchiseptica* 8	FCV 48
FHV 13	FCV/B. *bronchiseptica* 4	FHV/FCV/*C. felis* 4	FHV-1 42
B. bronchiseptica 11	FHV/*C. felis* 1	FHV/FCV/C. felis/*B. bronchiseptica* 2	*B. bronchiseptica* 26
C. felis 1	FHV/*B. bronchiseptica* 1		*C. felis* 8

diagnosis of FCV produced an ORF2 gene fragment of 467 bp. The PCR for the diagnosis of *B. bronchiseptica* produced a fragment of 284 bp (Figure 1). Moreover, the restriction digestion with *Hind*III of all *Chlamydophila* spp. positive samples gave the same pattern, specific for *C. felis* (Figure 2).

FCV, FHV-1, *B. bronchiseptica* and *C. felis* were detected alone or in association in 48, 42, 26 and 8 cats respectively, for a total of 75 positive animals for one or more pathogens.

Single infections were detected in 42/100 animals. In particular, 17 cats were positive for FCV, 13 for FHV-1, 11 for *B. bronchiseptica* and one for *C. felis*. Mixed infections were detected in 33/100 of

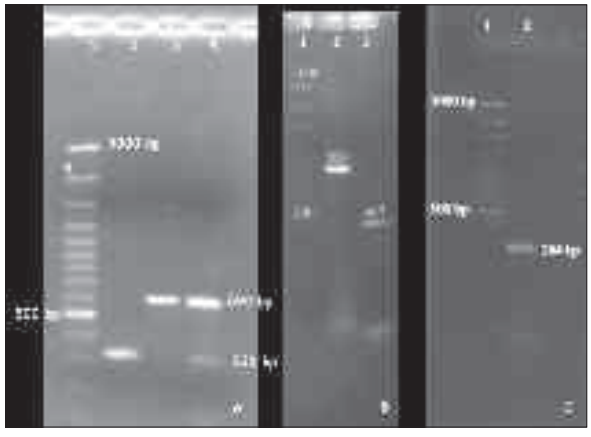

FIGURE 1 - *PCR products for FHV-1,* Chlamydophila *spp., FCV and* B. bronchiseptica. ***a**: Lane 1 - marker Gene Ruler™ 100 bp DNA Ladder PlusP (MBI Fermentas GmbH, Germany); Lane 2 - PCR for FHV-1; Lane 3 - PCR for* Chlamydophila *spp.; Lane 4 - Duplex PCR for FHV-1 and* Chlamydophila *spp.* ***b**: Lane 1 - marker Gene Ruler™ 100 bp DNA Ladder PlusP (MBI Fermentas GmbH, Germany); Lane 2 - PCR for FCV; Lane 3 - Nested PCR for FCV.* ***c**: Lane 1 - marker Gene Ruler™ 100 bp DNA Ladder PlusP (MBI Fermentas GmbH, Germany); Lane 2 - PCR for* B. bronchiseptica.

FIGURE 2 - *RFLP analysis for* C. felis *identification. Lane 1 - Marker Gene Ruler™ 100 bp DNA Ladder PlusP (MBI Fermentas GmbH, Germany); Lane 2 - PCR product for* Chlamydophila *spp.; Lane 3 - Specific pattern for* C. felis *after restriction analysis by* Hind*III.*

TABLE 3 - *PCR results correlated to the sample type.*

	PCR-positive samples				*Examined swabs*
Sample	*FHV-1*	*FCV*	*C. felis*	*B. bronchiseptica*	
Conjunctival swab	29	20	8	14	92
Pharyngeal swab	30	38	0	20	97

TABLE 4 - *Habitat of the tested cats.*

Habitat	*Positive cats*	*Negative cats*	*Total*
Domestic	8	18	26
Free-ranging	67	7	74
Total	75	25	100

TABLE 5 - *Age of the tested cats.*

Age	*Positive cats*	*Negative cats*	*Total*
≤12 months	42	10	62
>12 months	27	12	39
Unknown	6	3	12
Total	75	25	100

the examined cats. In detail, dual infections were detected in 13 animals for FHV-1/FCV, in one cat for FHV-1/*B. bronchiseptica*, in 4 cats for FCV/*B. bronchiseptica* and in one animal for FHV-1/*C. felis*. Multiple infections involved 4 animals for FHV-1/FCV/*C. felis* and 8 animals for FHV-1/FCV/*B. bronchiseptica*.

Simultaneous detection of all four pathogens were obtained in two cats.

Positive results for *C. felis* were obtained only from ocular swabs, whereas for FHV-1, FCV and *B. bronchiseptica* both types of specimen resulted positive alternatively (Table 3).

The animals came from different habitats. A higher proportion of positive cats (89,5%) were stray animals or had open access to the outside than negative cats (28%) (χ^2 36.6597; $p<0.05$) (Table 4). Moreover a higher proportion of positive cats (56%) were ≤12 month old than negative cats (40%) (χ^2 = 4,4335; $p<0.05$) (Table 5).

DISCUSSION

This study is the first attempt in Italy to evaluate the concurrent presence of major causative agents in URTD-affected cats. In accordance with previous surveys, FCV and FHV-1 were the most frequent respiratory pathogens. However, in this work a much higher detection rate for FCV and *B. bronchiseptica* was obtained with respect to previous reports (Pennisi *et al.,* 1999; Binns *et al.,* 1999; Binns *et al.,* 2000; Mochizuki *et al.,* 2000; Sykes *et al.,* 2001; Helps *et al.,* 2005). Although it is difficult to compare results among different studies, this discrepancy may be due either to the higher sensitivity of PCR assays we developed or to the features of the examined population which included only respiratory syndrome-affected cats. Moreover, the collected specimens could influence the results. In fact, for each animal conjunctival and pharyngeal swabs were collected to

increase the probability of identifying infected cats. The results show that more pharyngeal than conjunctival swabs were positive for FCV, FHV-1 and *B. bronchiseptica*. These data suggest that an appropriate diagnostic approach for detection of FCV, FHV-1 and *B. bronchiseptica* should include analysis of both types of samples.

Dual or multiple infections were detected in 33% of examined cats and in 56% of positive animals. Despite the small size of tested samples, the results demonstrate that the investigated pathogens are frequently associated in URTD-affected cats. In particular, the presence of *C. felis* infection, except in one cat, was always associated with FHV-1 infection. The detection of both pathogens is a common finding in the Japanese feline population (10.6%), whereas it is less frequent in Australia (0.6%) and USA (1.6%) (Nasisse *et al.,* 1993; Sykes *et al.,* 1999; Cai *et al.,* 2002). In contrast, *B. bronchiseptica* infection was not always associated with other viral infections, according to Binns *et al.,* (1999). The age and environment of the animals were recorded to identify hypothetical risk factors. The higher detection rate observed in cats with an age ≤12 months suggests that young animals are more susceptible to respiratory diseases, probably as consequence of their specific immunological condition. In fact, in cats living in colonies and/or outside and in accordance with previous survey reports (Binns *et al.,* 1999; Pennisi *et al.,* 1999; Binns *et al.,* 2000), an evident URTD predisposition was observed.

In conclusion, the PCR assays we developed are effective diagnostic tools to discriminate FCV, FHV-1, *C. felis* and *B. bronchiseptica* infections and to improve identification of URTD-affected animals. Moreover, compared to the traditional isolation assays, molecular analyses do not require viable organisms, thus simplifying collection, transportation and storage of the samples. This study shows a significant presence of the investigated pathogens in the examined area. Dual and multiple infections may be very common especially in young and free-ranging animals. Moreover, the primary agents of infection, such as FCV and FHV-1, are frequently associated with other less common pathogens, such as *B. bronchiseptica* and *C. felis*. The role of these pathogens in URTD insurgence cannot be considered secondary, suggesting the need for their inclusion in current diagnostic or vaccinal protocols.

ACKNOWLEDGEMENTS

We are grateful to Ottavio Palucci for the excellent technical assistance.

REFERENCES

BANNASCH, M.J., AND FOLEY, J.E. (2005). Epidemiologic evaluation of multiple respiratory pathogens in cats in animals shelter. *J. Feline Med. Surg.* **7**, 109-119.

BINNS, S.H., DAWSON, S., SPEAKMAN, A.J., CUEVAS, L.E., GASKELL, C.J., HART, C.A., MORGAN, K.L., AND GASKELL, R.M. (1999). Prevalence and risk factors for feline *B. bronchiseptica* infection. *Vet. Rec,* **144**, 575-580.

BINNS, S.H., DAWSON, S., SPEAKMAN, A.J., CUEVAS, L.E., HART, C.A., GASKELL, C.J., MORGAN, K.L., AND GASKELL, R.M. (2000). A study of feline upper respiratory tract disease with reference to prevalence and risk factors for infection with Feline Calicivirus and Feline Herpesvirus. *J. Feline Med. Surg,* **2**, 123-133.

CAI, Y., FUKUSHI, H., KOYASU, S., KURODA, E., YAMAGUCHI, T., AND HIRAI, K. (2002). An etiological investigation of domestic cats with conjunctivitis and upper respiratory tract disease in Japan. *Journal of Medical Veterinary Sciences.* **64**, 215-219.

COUTTS, A.J., DAWSON, S., BINNS, S., HART, C.A., GASKELL, C.J., AND GASKELL, R.M. (1996). Studies on natural transmission of *Bordetella bronchiseptica* in cats. *Vet. Microbiol.* **48**, 19-27.

DAWSON, S., RADFORD, A., AND GASKELL, R. (2004). Clinical update on feline respiratory pathogens. *In Practice.* **26**, 320-323.

DI FRANCESCO, A., CARELLE, M.S., AND BALZELLI, R. (2001). Evidenziazione del DNA di *Chlamydia pittaci* e Herpesvirus Felino tipo 1 mediante Polymerase Chain Reaction (PCR). *Summa.* **8**, 51-54.

DI MARTINO, B., MERIDIANI, I., AND MARSILIO, F. (2005). Allestimento di una PCR per la diagnosi delle infezioni da *B. bronchiseptica* del gatto. *Summa.* **12**, 11-14.

HELPS, C., REEVES, N., EGAN, K., HOWARD, P., AND HARBOUR, D. (2003). Detection of *Chlamydophila felis* and feline herpesvirus by multiplex real-time PCR analysis. **41**, 2734-2736.

HELPS, C.R., LAIT, P., DAMHUIS, A., BJORNEHAMMAR, U., BOLTA, D., BROVIDA, C., CHABANNE, L., EGBERINK, H., FERRAND, G., FONTBONNE, A., PENNISI, M.G., GRUFFYDD-JONES, T., GUNN-MORE, D., HARTMANN, K., LUTZ, H., MALANDAIN, E., MOSTL, K., STENGEL, C., HARBOUR, D.A.,AND GRAAT, E.A.M. (2005). Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, *Chlamydophila felis* and *Bordetella bronchiseptica* in cats: experience from 218 European catteries. *Vet. Rec.* **156**, 669-673.

HOLST, B.S., BERNDTSSON, L.T., AND ENGLUND, L. (2005). Isolation of feline herpesvirus-1 and feline calicivirus from healthy cats in Swedish breeding catteries. *J. Feline Med. Surg.* **7**, 325-331.

HOSKINS, J.D., WILLIAMS, J., ROY, A.F., PETER, J.C., AND MCDONOUGH, P. (1998). Isolation and characterization of *Bordetella bronchiseptica* from cats in southern Louisiana. *Vet. Immunol. Immunopathol*. **65**, 173-176.

JACOBS, A.A.C., CHALMERS, W.S.K., PASMAN, J., VAN VUGT, F., AND CUENEN, L.H. (1993). Feline bordetellosis: challenge and vaccination studies. *Vet. Rec*. **133**, 260-263.

MARSILIO, F., DI MARTINO, B., AGUZZI, I., AND MERIDIANI, I. (2004). Duplex Polymerase Chain Reaction assay to screen for feline Herpesvirus-1 and *Chlamydophila* spp. in mucosal swabs from cats. *Vet. Res. Comm.* **28**, 295-298.

MARSILIO, F., DI MARTINO, B., DECARO, N., AND BUONAVOGLIA, C. (2005). A novel nested PCR for the diagnosis of calicivirus infections in the cat. *Vet. Microbiol.* **105**, 1-7.

MOCHIZUKI, M., KAWAKAMI, K., HASHIMOTO, M., AND ISHIDA, T. (2000). Recent epidemiological status of feline upper respiratory infections in Japan. *Journal of Medical Veterinary Sciences,* **62**, 801-803.

NASISSE, M.P., GUY, J.S., STEVENS, J.B., ENGLISH, R.V., AND DAVIDSON, M.G. (1993). Clinical and laboratory findings in chronic conjunctivitis in cats. *JAVMA.* **203**, 834-837.

PASMANS, F., ACKE, M., VANROBAEYS, M., AND HAESEBROUCK, F. (2001). Prevalence of *B. bronchiseptica* infections in cats from different environments. *Vlaams Diergeneeskunding Tijdschrift.* **70**, 124-126.

PENNISI, M.G., FERA, M.T., MASUCCI, M., DE MAJO, M., AND CARBONE, M. (1999). Isolation of *B. bronchiseptica* in cats: Clinical and epidemiological evaluation. *Proceeding of the IAIEV*. November 18 to 20, Palermo, Italy.

SYKES, J.E., ANDERSON, G.A., STUDDERT, V.P., AND BROWNING, G.F. (1999). Prevalence of feline *Chlamydia psittaci* and feline herpesvirus 1 in cats with upper respiratory tract disease. *J. Vet. Intern. Med.* **13**, 153-162.

SYKES, J.E., ALLEN, J.L., STUDDERT, V.P., AND BROWING, G.F. (2001). Detection of feline calicivirus, feline herpesvirus 1 and *Chlamydia psittaci* mucosal swabs by multiplex RT-PCR/PCR. *Vet. Microbiol.* **81**, 95-108.

SYKES, J.E. (2001) Feline upper respiratory tract pathogens: Herpesvirus-1 and Calicivirus. *Compendium on Continuing Education for the Practicing Veterinarian*. **23**, 166-175.

SYKES, J.E. (2005). Feline Chlamydiosis. *Clinical Techniques in Small Animal Practice.* **20**, 129-134.

WELSH, R.D. (1996). *Bordetella bronchiseptica* infections in cats. *J. Am. Anim. Hosp. Assoc.* **32**, 153-158.