aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Animal and Veterinary Advances

ISSN 1683-9919
DOI: 10.3923/ajava.2017.109.114

Research Article
Detection of Multi-drug Resistant Bacterial Recovered in a Community Animal Control Setting

[1,2]Pongpon Homkong, [1,3]Jeff B. Bender, [2]Pirun Chutipongvivate, [1]Veerasak Punyapornwithaya, [1]Terdsak Yano and [1]Khwanchai Kreausukon

[1]Faculty of Veterinary Medicine, Chiang Mai University, Maehia, Muang, 50100 Chiang Mai, Thailand
[2]Chiang Mai Municipality, Veterinary Services, 1 Wangsincome Road, Muang, 50300 Chiang Mai, Thailand
[3]College of Veterinary Medicine, University of Minnesota, 136F ABLMS, 1354 Eckles Avenue Street, Paul, Minnesota, 55108, United State of America

Abstract
Objective: The intent of this study was to characterize the potential bacterial contamination before and after sterilization in a limited resource setting and re-assess good surgical practices. This included the monitoring of antimicrobial susceptibility of recovered isolates, which have bearing on optimal antimicrobial use. **Materials and Methods:** Samples were collected from three surgical sites on the patient (two before cutting and one after), surgeon's hand and pooled sample from surgical instruments in sterilization process. Antimicrobial susceptibility was performed on recovered bacteria. Descriptive analyses were done to compare between isolate recovery between sites and susceptibility findings. **Results:** Dogs enrolled in the study ranged in age from 0.5-6 years (mean 2.4). Bacterial isolates were recovered from 28 (93%) dogs; 17 (57%) surgeon's hands and 8 (27%) pooled surgical equipment swabs. Eighty two unique bacterial isolates were recovered from 150 collected samples. Of the 82 isolates, 18 were identified as *Staphylococcus* sp. and 64 were identified as *Bacillus* sp. and *Staphylococcus* sp., were generally susceptible to amoxicillin/clavulanic acid, cephalexin, chloramphenicol and gentamycin. Thirteen different antimicrobial resistance patterns were observed for *Staphylococcus* sp. Four isolates were pan-sensitive. About 10 of 18 isolates were resistant to three of more classes of antibiotics. **Conclusion:** Reproductive surgical sterilization is an important tool in efforts to control pet overpopulation, even in limited resource settings. With ongoing concerns about emerging antibiotic resistance, implementation of optimum aseptic technique is needed. Findings from this study support standard and appropriate surgical protocols in dog control sterilization in limited resource settings which supports optimal antibiotic use policies.

Key words: Antibiotic resistance, multi-drug resistance, dog population control, sterilization, stray dogs, bacterial contamination, *Staphylococcus* sp.

Received: September 30, 2016 Accepted: November 28, 2016 Published: January 15, 2017

Citation: Pongpon Homkong, Jeff B. Bender, Pirun Chutipongvivate, Veerasak Punyapornwithaya, Terdsak Yano and Khwanchai Kreausukon, 2017. Detection of multi-drug resistant bacterial recovered in a community animal control setting. Asian J. Anim. Vet. Adv., 12: 109-114.

Corresponding Author: Khwanchai Kreausukon, Faculty of Veterinary Medicine, Chiang Mai University, Thailand Tel/Fax: +66 53 948002

Copyright: © 2017 Pongpon Homkong *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stray and feral dogs are linked to serious human health (i.e., rabies), animal health and welfare problems[1]. In many countries, dog population control is a complex issue which is influenced by socio-economic, political and religious factors. The World Organization for Animal Health (OIE) recognizes the importance of controlling dog populations without causing unnecessary animal suffering[1]. It also recognizes the important role of veterinary care in controlling animal populations, preventing zoonotic diseases and ensuring animal welfare.

In 2014 in the Chiang Mai municipal area, there are an estimated 6624 owned dogs and 612 free roaming or temple dogs. In the downtown area, these free roaming or discarded dogs scrounge for food near tourist areas, food markets and city dumps[2]. City officials receive numerous complaints regarding these free or stray dogs in the city.

As a means to control the stray dog populations, the "Companion Animal Control Project of Chiang Mai Municipality" has developed a program that includes public education, dog registration, unwanted dog adoptions and dog sterilization. According to the guidelines of International Companion Animal Management Coalition[3], sterilization practices needs to be done by a veterinarian under aseptic conditions with appropriate anesthesia and pain management. Appropriate aseptic technique includes surgical prep of the patient, sterilization of surgical instruments and maintenance of asepsis during surgery. Previous studies have found that, in surgeon practice if using a hand rub (Hand rubbing) alcohol with ethanol after washing with mild soap can reduce the amount of bacteria as well and found fewer bacteria at the end of surgery than hand scrubbing[4,5]. This includes monitoring for surgical wound infections post-operatively[6] that may have some problem from surgical[7]. Inflammation post surgery infection that is found in a third of the problems encountered by surgical sterilization[8]. The primary problem from contaminated is a wound infection[9] *Staphylococcus intermedius* is a common problem of skin infection in dogs[10,11]. *Steptococcus* spp. and *Staphylococcus* spp., are the microbe that can be found to infect on the skin and the subcutaneous layer and become to be an abscess. Other infections that can cause dermatitis is *Blastomyces dermatitidis* and *Sporothix schenckii*[12]. Moreover, lock jaw and pyoderma are caused by skin infection, lock jaw is caused by *Costridium tetani* and pyoderma can caused by many bacterial include *Corynebacterium* spp., *Staphylococcus aureus* and *Staphylococcus pyogeneus*[13].

The intent of this study was to characterize the potential bacterial contamination before and after sterilization in a limited resource setting and re-assess good surgical practices. This included the monitoring of antimicrobial susceptibility of recovered isolates, which have bearing on optimal antimicrobial use. The goal was to provide the "Best practices" in a limited funded dog control program ("Companion Animal Control Project" of Chiang Mai Municipality). This information will useful for other local municipalities and veterinarians involved in dog population control programs.

MATERIALS AND METHODS

Enrolled animals: This was a voluntary program supported by Chiang Mai University College of Veterinary Medicine and the "Companion Animal Control Project of Chiang Mai Municipality" between January, 2015 and April, 2015. Thirty healthy female owned dogs, between the ages of 1-10 years were enrolled (The protocol of this study was improved by Animal Ethic Committee, Faculty of Veterinary Medicine, Chiang Mai University to use animal enrolled in study (No. S30/2557)). These dogs varied by breed and there was no history of prior antibiotic use and all of the data were managed with commercial available software program (Excel 2013, Microsoft Corp, Redmond, Wash). Surgical procedures were done by Veterinary Services of the Chiang Mai City Municipality.

Sample collection: Sample collection occurred from February through April, 2015. There were 4 surgeons performing ovariohysterectomies at the sterilization room of the Veterinary section, Chiang Mai Municipality. Four sets of surgical instruments were sanitized in alkyl dimethyl ammonium chloride (Umonium[38], Huckert's International, Nivelles, Belgium) for at least 15 min. Cotton swabs were used to collect samples from three surgical sites (two before cutting and one after), surgeon's hand and pooled sample from surgical instruments. After surgical prep with betadine scrub and an alcohol wipe, samples were obtained from the (1) Area surrounding the incision (ASR) within the prepped area, (2) The incisional site before surgery (INB) and (3) The incision site after closure and suturing (INA). A swab sample from the surgeon's hand was collected after scrubbing (SHD). A pooled swab sample was collected from the following instruments including the rat toothed forceps, tissue scissors, Allis tissue forceps and the needle holder (INS).

Bacteriological methods: The samples were immediately inoculated on sheep blood agar and Mac-Conkey agar. Agar plates were incubated at 37°C for 24-48 h at the Central Laboratory of Faculty of Veterinary Medicine, Chiang Mai

University. If multiple colonies were present up to three colonies were characterized. This was more common from the dog skin collection sites. Bacterial identification was performed by examining colony type and morphology, catalase test and Gram stain. Biochemical tests were used to confirm *Bacillus* sp. and *Staphylococcus* sp.[14].

Antimicrobial susceptibility: Antimicrobial susceptibility was performed by disc diffusion according to Clinical and Laboratory Standards Institute[15] guidelines in Mueller-Hinton agar (Oxoid). The following 12 antimicrobials were used: Ampicillin (AMP: 10 µg), amoxicillin (AML: 10 µg), amoxicillin and clavulanic acid (AMC: 30 µg, amoxicillin: 20 µg and clavulanic acid: 10 µg), cefotaxim (CTX: 30 µg), cephalexin (CEP: 30 µg), (chloramphenicol ©: 30 µg), ciprofloxacin (CIP: 5 µg), doxycycline (DO: 30 µg), enrofloxacin (ENR: 5 µg), gentamycin (CN: 10 µg), penicillin (P: 10 U), streptomycin (STP: 25 µg), sulfametoxazole and trimethoprim (SXT: 23.5 and 1.25 µg).

Analysis: Descriptive summary statistics were provided to characterize the frequency of isolation by site and describe the resistance profiles of *Staphylococcus* and *Bacillus* isolates. When multiple isolates were available by site with similar susceptibility profiles, only one was used in the summary analysis. The chi-square test was used when appropriate to compare bacterial isolates among before operation procedure and after operation procedure. Analyses were performed with free available statistical software (R program, R version 3.3.1, The R Foundation of Statistical Computing).

RESULTS

Age was available on 27 of 30 dogs. Dogs ranged in age from 0.5-6 years (mean 2.4). Bacterial isolates were recovered from 28 (93%) dogs; 17 (57%) surgeon's hands and 8 (27%) pooled surgical equipment swabs. Multiple different isolates were recovered from the area around the surgery site. For the analysis only unique isolate patterns were included based on the susceptibility profile. Eighty two unique bacterial isolates were recovered from 150 collected samples (Table 1). Of the 82 isolates, 18 were identified as *Staphylococcus* sp. and 64 were identified as *Bacillus* sp. In three surgeries, *Staphylococcus* sp., was recovered from multiple samples (i.e., surgeon hands and the skin during the same surgery). In 24 surgeries, *Bacillus* sp., was recovered from one or more sites. Total of 7 isolates (n = 7) were recovered from the disinfected surgical equipment. A comparison of frequency of bacterial recovery from the surgical incision site before and after the surgical procedure was similar (p = 0.8).

The susceptibility of *Staphylococcus* sp. and *Bacillus* sp., isolates to individual antimicrobials is shown in Table 2 and 3. *Staphylococcus* sp., were generally susceptible (e.g., ≤85%) to amoxicillin/clavulanic acid, cephalexin, chloramphenicol and gentamycin. *Staphylococcus* isolates were resistant (e.g., <50%) to ampicillin, penicillin, streptomycin and sulfametoxazole/trimethoprim. *Bacillus* sp., isolates were susceptible (e.g., ≤85%) to most antibiotics except penicillin (72% susceptible).

Resistance profiles from the *Staphylococcus* isolates are summarized in Table 4. Thirteen different antimicrobial resistance patterns were observed for *Staphylococcus* sp. Four isolates were pan-sensitive. Ten of 18 isolates were resistant to three of more classes of antibiotics. Seventeen different antimicrobial resistance patterns were observed for *Bacillus* sp., 35 (55%) were susceptible to all antibiotics in the panel. Eleven isolates only had reduced susceptibility to

Table 1: Recovery of *Staphylococcus* spp. and *Bacillus* spp. by site

	Staphylococcus spp.		*Bacillus* spp.	
Sample site (n = 30)	n	%	n	%
Area surrounding incision	6	20	21	70
Incision site before cut	0		16	53
Incision site after closure	3	10	10	33
Surgeon hands	8	27	10	33
Pooled instrument	1	3	7	23

Table 2: Antibiotic *Staphylococcus* sp., susceptibility of recovered isolates

Antibiotic	All isolates (n = 18) (%)
Amoxicillin (AML)	10 (56)
Amox and clauvulanic acid (AMC)	16 (89)
Ampicillin (AMP)	8 (44)
Cephalexin (CEP)	16 (89)
Chloramphenicol (C)	16 (89)
Ciprofloxacin (CIP)	12 (67)
Doxycycline* (DO)	15 (83)
Enrofloxacin (ENR)	15 (83)
Gentamycin (CN)	17 (94)
Penicillin (P)	6 (33)
Streptomycin (STP)	7 (39)
Sulfametaxazole and trimethoprim (SXT)	7 (39)

*Susceptibility done on 17 isolates

Table 3: Antibiotic *Bacillus* sp., susceptibility of recovered isolates

Antibiotic	All isolates (n = 64) (%)
Amoxicillin (AML)	56 (88)
Amox and clauvalanic acid (AMC)	60 (94)
Ampicillin (AMP)	59 (92)
Cephalexin (CEP)	61 (95)
Chloramphenicol (C)	62 (97)
Ciprofloxacin (CIP)	61 (95)
Doxycycline (DO)	63 (98)
Enrofloxacin (ENR)	62 (97)
Gentamycin (CN)	62 (97)
Penicillin (P)	46 (72)
Streptomycin (STP)	59 (92)
Sulfametaxazole and trimethoprim (SXT)	54 (84)

Table 4: Antimicrobial resistant patterns of *Staphylococcus* spp., isolates (n = 14)*

Resistant phenotype	Frequency
SXT	1
CIP, STP, SXT	1
P, STP, SXT	1
AML, AMP, P, STP	1
AMP, C, P, STP	1
AML, AMP, C, P, STP	1
AML, AMC, AMP, P, SXT	2
AMP, CIP, P, STP, SXT	1
AML, AMP, CEP, CIP, P, STP, SXT	1
AML, CIP, ENR, P, STP, SXT	1
AMP, CIP, ENR, P, STP, SXT	1
AML, AMP, CEP, DO, P, STP, SXT	1
AML, AMP, CIP, DO, ENR, P, STP, SXT	1

*Four isolates were pan-sensitive

penicillin. Five *Bacillus* sp., isolates were resistance to 3 or more different antimicrobial classes.

DISCUSSION

This study demonstrated that even in settings with limited resources such as city animal control programs, aseptic practices can be followed limiting the potential contamination of surgical sites[16,17]. These surgeries were done in a single room, where preparation including hair clipping, skin disinfection, surgery and surgical recovery. To the researchers knowledge, none of the dogs developed post-surgical infections. Surgeons need to pay attention and follow standard procedures for surgical preparation including use of a surgical hand scrub, wearing sterile gloves and practicing sterile technique to reduce bacterial contamination. Other aspects of operating room etiquette include limiting access of personnel to the surgical room during procedures and maintaining flow from “Clean” to “Dirty” areas.

This study also provides insight into the degree of bacterial resistance observed among commensal bacteria in a Thailand dog population control program. The identification of *Staphylococcus* with reduced susceptibility to a number of different classes of antibiotics is evidence that multi-drug resistant infections are a present day reality[18]. Both *Staphylococcus* and *Bacillus* were readily recovered from the area around the incisional site irrespective of previous disinfection. *Staphylococcus* sp., is a normal commensal of the canine skin[19,20] and in some studies 46% of skin samples readily recovered *Staphylococcus*[9,21]. Also, in a study evaluating three different disinfectant protocols, researchers recovered *Staphylococcus intermedius* in 9.2% and *Staphylococcus* sp., in 8.4% of samples collected after antisepsis[17]. This is not surprising; the surgery site is not a sterile environment. This fact re-emphasizes the value of aseptic technique for routine procedures. Some of the recovered bacteria were multi-drug resistant, especially the *Staphylococcus* isolates[22,23]. This may reflect the emergence of multi-drug resistant *Staphylococcus pseudintermedius*[24,25] and healthy dogs can be a reservoir of antimicrobial-resistant bacteria which may be transferred to surgeon and their owners[26].

To prevent nosocomial and zoonotic transmission, incorporation of infection prevention practices is important for all veterinary settings just like in human health-care setting. These efforts include the comprehensive promotion of infection prevention practices in companion animal settings including animal shelters[27]. The thoughtful and appropriate use of antibiotics as part of antibiotic stewardship programs should be incorporated in these plans[28]. Generally, in less than ideal settings antibiotics are often prescribed before and after the surgical procedure. The use of antibiotics is generally contra-indicated for the healthy patient undergoing routine, aseptic procedures. Findings from the study have implications for standard and appropriate surgical protocols in dog control sterilization in such settings and the optimal use of antibiotics to prevent veterinary-setting acquired infections. As a result of this study, the Veterinary Services of the City of Chiang Mai Municipality changed their policy of providing antibiotics post surgically.

In pet population control efforts globally, reproductive surgical sterilization is an important tool in broad efforts to control pet over-population[29]. Adherence to aseptic technique is possible in limited resource settings. With current concerns about the transfer and transmissibility of antibiotic resistant elements, it is important to utilize antibiotics optimally and review practices even in sub-optimal conditions.

CONCLUSION

Reproductive surgical sterilization is an important tool in efforts to control pet overpopulation and also to eliminate rabies virus, even in limited resource settings. With ongoing global concerns about emerging antibiotic resistance, implementation of optimum aseptic technique is needed. Findings from this study support standard and appropriate surgical protocols in dog control sterilization in limited resource settings that can uses where similar places and which supports optimal antibiotic use policies.

ACKNOWLEDGMENTS

We appreciate the support from the “Companion Animal Control Project of Chiang Mai Municipality” and also the

Central Laboratory of Faculty of Veterinary Medicine, Chiang Mai University for the laboratory helpful.

REFERENCES

1. OIE Terrestrial Animal Health Standards Commission, 2009. Guidelines on stray dog population control. Capture 7.7., Terrestrial Animal Health Standards Commission, September 2009, pp: 313-621.
2. Kongtueng, P., U. Sankaewthong, J. Thaiklang, P. Pilin, W. Tangpokanon, W. Wongkalasin and S. Rojanasthien, 2006. Stray dog's population in Maung district, Chiang Mai. Chiang Mai Vet. J., 4: 57-62.
3. ICAM., 2016. Humane dog population management guidance. International Companion Animal Management Coalition. http://www.icam-coalition.org/
4. Parienti, J.J., P. Thibon, R. Heller, Y. le Roux and P. von Theobald *et al*., 2002. Hand-rubbing with an aqueous alcoholic solution vs traditional surgical hand-scrubbing and 30-day surgical site infection rates: A randomized equivalence study. J. Am. Med. Assoc., 288: 722-728.
5. Picheansathian, W., 2004. A systematic review on the effectiveness of alcohol-based solutions for hand hygiene. Int. J. Nurs. Prac., 10: 3-9.
6. May, E.R., 2006. Bacterial skin diseases: Current thoughts on pathogenesis and management. Vet. Clin. North Am. Small Anim. Pract., 36: 185-200.
7. Turk, R., A. Singh and J.S. Weese, 2015. Prospective surgical site infection surveillance in dogs. Vet. Surg., 44: 2-8.
8. Berzon, J.L., 1979. Complications of elective ovariohysterectomies in the dog and cat at a teaching institution: Clinical review of 853 cases. Vet. Surg., 8: 89-91.
9. Rodkhum, C., T. Reudeejai, R. Snonguthai, S. Pongsriwat, S. Kumlangpeath and P. Karkrai, 2007. Bacterial from skin lesions of dogs and cats and their antimicrobial susceptibility focused on resistance to cephalosporins and fluoroquinolones of *Staphylococcus intermedius*. Proceedings of the 33th Conference on Veterinary and Companion Animal, October 31-November 2, 2007, Bangkok, pp: 17-20.
10. Muller, G.H., R.W. Kirk, D.W. Scott, W.H. Miller and C.E. Griffin, 1983. Small Animal Dermatology. W.B. Saunders, Philadelphia, pp: 197-213.
11. Ihrke, P., 1990. Bacterial Infection of the skin. In: Infectious Diseases of the Dog and Cat, Greene, C.E. (Ed.)., 1st Edn., WB Saunders, Philadelphia, pp: 72-79.
12. Carter, G.R. and J.R. Cole, Jr., 1990. Diagnostic Procedures in Veterinary Bacteriology and Mycology. 5th Edn., Academic Press, New York, USA., pp: 221-229.
13. Ganiere, J.P., C. Medaille and C. Mangion, 2005. Antimicrobial drug susceptibility of *Staphylococcus intermedius* clinical isolates from canine pyoderma. J. Vet. Med. Ser. B, 52: 25-31.
14. Shimeld, L.A. and A.T. Rodgers, 1999. Essentials of Diagnostic Microbiology. Delmar Publishers, Albany, ISBN: 9780827373884, Pages: 690.
15. Clinical and Laboratory Standard Institute, 2009. Performance Standards for Antimicrobial Susceptibility Testing: 20th Informational Supplement. Clinical and Laboratory Standard Institute, Wayne, PA., USA.
16. Okken, A.C., S.J. Dieleman and I.V.D. Gaag, 1981. [Gynaecological complications following ovariohysterectomy in dogs]. Tijdschr. Diergeneeskd., 106: 1142-1158.
17. Akinrinmade, J.F. and O.D. Eyarefe, 2012. Enterologic and gynaecologic complications of ovariohysterectomy in the bitch. Nig. Vet. J., 33: 571-576.
18. Guardabassi, L., S. Schwarz and D.H. Lloyd, 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria: Review. J. Antimicrob. Chemother., 54: 321-332.
19. Lambrechts, N.E., K. Hurter, J.A. Picard, J.P. Goldin and P.N. Thompson, 2004. A prospective comparison between stabilized glutaraldehyde and chlorhexidine gluconate for preoperative skin antisepsis in dogs. Vet. Surg., 33: 636-643.
20. Harvey, R.G. and D.H. Lloyd, 1994. The distribution of *Staphylococcus intermedius* and coagulase negative staphylococci on the hair, skin surface, within the hair follicles and on the mucous membranes of dogs. Vet. Dermatol., 5: 75-81.
21. Rodkhum, C., R. Maugkeaw, P. Makum, S. Ruedeejai, R. Snonguthai and S. Pongsiwat, 2009. Prevalence and antimicrobial resistance of Staphylococcus intermedius group (SIG) isolated from skin lesions of dogs in Thailand. Proceedings of the 8th Chulalongkorn University Veterinary Science Annual Conference, April 3, 2009, Bangkok, pp: 91.
22. Werckenthin, C., M. Cardoso, J.L. Martel and S. Schwarz, 2001. Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus* and canine *Staphylococcus intermedius*. Vet. Res., 32: 341-362.
23. Vanni, M., R. Tognetti, C. Pretti, F. Crema, G. Soldani, V. Meucci and L. Intorre, 2009. Antimicrobial susceptibility of *Staphylococcus intermedius* and *Staphylococcus schleiferi* isolated from dogs. Res. Vet. Sci., 87: 192-195.
24. Vincze, S., A. Paasch, B. Walther, C. Ruscher, A. Lubke-Becker, L.H. Wieler and K. Barbara, 2010. Multidrug-and methicillin resistant *Staphylococcus pseudintermedius* as a cause of canine pyoderma: A case report. Berl. Munch. Tierarztl. Wochenschr., 123: 353-358.
25. Van Duijkeren, E., M. Kamphuis, I.C. van der Mije, L.M. Laarhoven, B. Duim, J.A. Wagenaar and D.J. Houwers, 2011. Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet. Microbiol., 150: 338-343.

26. Todsaparameeyanarujee, T., P. Rongdon, J. Thongyoo, D. Umnuayonvaree and N. Tuncharoen *et al*., 2015. Methicillin resistant and multiple antimicrobial resistant staphylococci in dogs and owners. Proceeding of the 53rd Kasertsart University Annual Conference, February 3-6, 2015, Thailand, pp: 1045-1052.
27. Mostl, K., H. Egberink, D. Addie, T. Frymus and C. Boucraut-Baralon *et al*., 2013. Prevention of infectious diseases in cat shelters: ABCD guidelines. J. Feline Med Surg., 15: 546-554.
28. Bender, J.B., T.F. Barlam, R.P. Glore, N. Gumley and S.E. Grayzel *et al*., 2015. The AVMA task force for antimicrobial stewardship in companion animal practice responds. J. Am. Vet. Med. Assoc., 246: 727-728.
29. ACC&D., 2013. Contraception and fertilities control in dogs and cats: A report of the alliance for contraception in cats and dogs (ACC&D). Alliance for Contraception in Dog and Cat, February 2013.