Journal of Animal and Veterinary Advances 7 (4): 450-455, 2008
ISSN: 1680-5593

Prevalence of Serum Antibodies Against Six Leptospira Serovars in Sheep in Tabriz, Northwestern Iran

[1]Mohammad Tooloei, [3]Golamreza Abdollapour, [2]Hamid Karimi and [4]Ali Hasanpor
[1]Department of Clinical Sciences, [2]Department of Basic Sciences,
Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[3]Department of Clinical Sciences, Faculty of Veterinary Medicine,
University of Tehran, Tehran, Iran
[4]Department of Clinical Sciences, Faculty of Veterinary Medicine,
Tabriz Islamic Azad University, Tabriz, Iran

Abstract: Leptospirosis is an infectious zoonosis and its prevalence in sheep is unknown in Tabriz, northwestern Iran. Rodents and wildlife are a major source of infection that discharges the bacteria from their urine. The aims of the survey were evaluation of the seroprevalence of Leptospiral infection in sheep and the relationship between seroprevalence and animal and environmental factors in Tabriz. In this study, 359 serum samples randomly collected from sheep slaughtered in Tabriz industrial slaughterhouse since December 2004 to November 2005. Sera were stored at -20°C until use. They were initially screened at serum dilution of 1:100 against 6 live antigens of *Leptospira interrogans* serovars *pomona*, *canicola*, *hardjo*, *ballum*, *icterohaemorrhagiae*, *grippotyphosa* using the microscopic agglutination test (MAT). The samples were considered positive if ≥50% of agglutination of leptospire in a dilution tests serum of ≥l: 100 were observed. Sera with positive results were titrated against reacting antigens in serial 2-fold dilutions from l: 100 to 1:800. 66 serums (18/4%) at dilution ≥1:100 were seropositive against 1 or 2 of serovars. *Grippotyphosa* and *canicola* were detected as the most prevalent serovars with 39.7 and 30.2%, respectively. Prevalence rates of other serovars were 16.4% for *pomona*, 8.2% for *hardjo* and 5.5% for *icterohaemorrhagiae*. All of samples were seronegative for *ballum*. Statistical analysis of the results showed that the rates of the infection in the autumn (37%) and spring (21%) were significantly higher than the other seasons ($0.001<p<0.005$). The rate of the infection has been statistically increased with the aging ($0.001<p<0.005$) and the animals with 3 and 4 pair's permanent teeth (4-5 years old) had the highest infection rates. The infection rate in the female animals group was higher than the males ones ($0.01<p<0.05$). Thus, the serological infection rate in sheep in tabriz is relatively high and consequently the preventive methods must be applied to prevention of the spread of disease and its transmission to the human and other farm animals.

Key words: Leptospirosis, seroprevalence, sheep, MAT, Tabriz

INTRODUCTION

Leptospirosis is an important infectious disease of livestock animals and humans caused by the pathogenic leptospires which are classified into one species of *Leptospira interrogans* containing over 212 serovars. The infection has a worldwide distribution (Radostitis *et al*., 2007). The distribution of isolates and prevalence of serovars varies according to geographical regions and countries and depends on environmental and host factors. The source of the infection is an infected animal which contaminates pasture, drinking water and feed by infective urine, aborted fetuses and uterine discharges (Carter and Chengappa, 1991; Quinn *et al*., 2002). The infection is particularly, important due to causing abortion, stillbirth, mastitis, milk drop syndrome, occurrence of the acute disease and economic losses in farm animals (Saglam *et al*., 2007). Considering that the infection is usually developed by the native serovars, identification of the serovars in each region is very important in epidemiology of the infection in the same region (Cerri *et al*., 2003). Urine is the chief source of contamination because animals, even after clinical recovery, may shed leptospirae in the urine for long periods and act as carriers (Quinn *et al*., 2002). Leptospirosis is classified into 2 broad categories: host-adapted and non host-adapted. An animal infected with a host-adapted serovar of the organism, is

Corresponding Author: Mohammad Tooloei, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

a maintenance or reservoir host. Cattle are the maintenance host for some of the serovars, thus serological surveys of cattle in the world has found that relatively high percentages of the sera had antibodies against numerous leptospiral serovars, but sheep has been accepted as accidental or incidental hosts for the most leptospiral serovars (Bulu *et al*., 1990; Radostitis *et al*., 2007). However persistent leptospiruria due to L. hardjo in sheep where no contact with cattle has occurred (Radostitis *et al*., 2007) and also widespread leptospiral infection in merino rams in Australia, suggest that sheep may be a maintenance host at least for some of the serovars such as hardjo (Ellis *et al*., 1994). This could complicate control of the infection in cattle and also the infected sheep are a potential zoonotic risk to abattoir worker, sheep farmer and shearers which previously had not been considered (Ellis *et al*., 1994).

Considering that the high leptospiral seroprevalence rates of the cattle and buffalos in previous studies in Iran and East Azarbaijan province (Firouzi and Vandyousefi, 2000; Hasanpour *et al*., 2007; Shoaei, 1993) and with attention to the fact that sheep are usually in contact with cattle directly or indirectly in the most regions of the province, therefore this is predicted that sheep may be one of the important animals in epidemiology of the infection in Iran. Prevalence of leptospiral infection in sheep was unknown in Tabriz.

The purposes of the survey were to determine the seroprevalence rate of the leptospiral infection, to determine the titer of the antibodies against to different Leptospira serovars and evaluation of the relationship between seroprevalence and animal and environmental factors in sheep slaughtered in Tabriz, Iran.

MATERIALS AND METHODS

Animals and samples: The minimum sample size on the basis of the 95% confidence rate and the reviewed prevalence rates of other studies as well as the confounding rate equal to 5% was determined 359 sheep. Blood samples were stratified randomly taken from 365 sheep slaughtered in industrial slaughterhouse of Tabriz since December 2004 to November 2005. Ten mililiter of blood was collected from jugular vein of each sheep. None of animals had been vaccinated against leptospires. The animals were classified in to 5 age based groups, without permanent teeth and with 1, 2, 3 and 4 pair's permanent teeth. At the time of blood collection, all the animals appeared healthy with no clinical sign suggestive of leptospirosis. The samples were allowed to clot and centrifuged for 10 min at 2500×g after centrifugation; the serum was removed and stored at -20°C until use.

Serology: The sera were tested for antibodies against six live antigens of leptospira interrogans (*L. interrogans* serovar *grippotyphosa*, *icterohaemorrhagiae*, *hardjo*, *pomona*, *canicola* and *ballum* with regarding to the previous surveys in Iran) using Microscopic Agglutination Test (MAT) in leptospiral research laboratory, University of Tehran. EMJH- base 23% solutions (PH = 7.4) used for preparation of the different dilutions of sera (1:50 to 1:800). Adequate amounts of the 7-10 days old pure and without secondary contaminated leptospira cultures were being used as antigens. Load of the organisms was evaluated by a dark field microscope. The MAT was performed according to the methods of OIE (2000): sera were initially screened at a dilution of 1:100 against these antigens. At first, a serum dilution at 1:50 was made and a volume equal to the diluted serum volume of each antigen was added to each well of micro-titration plates, making the final serum dilution of 1:100. The micro-titration plates were incubated at 29°C for 2 h. The plates were then examined by dark-field microscopy. Results were considered positive when ≥50% of agglutination of leptospires at the test serum dilution of ≥1:100 were observed (OIE, 2000). Sera with positive results were titrated against reacting antigens in serial 2-fold dilutions from 1:100 to 1:800.

Statistical analysis: Results were statistically analyzed using the software SPSS 11.5 with the aid of Chi-Square and Fishers exact tests with 95% confidence interval to assess the association between the infection rates and age, sex and season.

RESULTS

Antibody prevalence's, as determined by positive results at a 1:100 dilution or higher, against one or more studied serovars was 18.4% (66/359) (Table 1).

Results of MAT: The highest number of reactors was for serovar *grippotyphosa* (39.7%) followed by *canicola* (30.2%), *pomona* (16.4%), *hardjo* (8.2%) and *icterohaemorrhagiae* (5.5%). Antibody against more than one serovars were found in seven (10.6%) sera of seropositive ones, so that mixed infection of *grippotyphosa* and *canicola*, *grippotyphosa* and *pomona* were seen in 5 and 2 sera, respectively. All of the samples were seronegative for serovar ballum (Table 2).

There are 73 recognizable Antibody titers from the all of 66 seropositive samples. The majority of titre levels were 1:100 for all serovars and the frequency of 1:100, 1:200 and 1:400 dilutions were 78.2% (57/73), 19.1% (14/73) and 2.7% (2/73), respectively (Table 2).

The distribution of the infection concerning varies seasons, comparison of infection rates between the seasons and the results of the statistical analyses are described in Table 3 and 4.

Statistical analysis of the results showed that the rates of the infection in the autumn (37%) and spring (21%) were significantly higher than the other seasons ($0.001<p<0.005$).

Seropositivity for Leptospirosis in the studied animals according to age (permanent teeth pairs) and sex is shown in Table 5.

Table 1: Seroprevalence of Leptospirosis in sheep slaughtered in Tabriz industrial Abattoir-Iran

	No. of tested	No. of positive[a] (f)	Prevalence (%)
Serum	359	66	18.4

a: Results of MAT

Table 2: Distribution of serovar specific antileptospiral antibodies and their titration in seropositive sheep

Serovar	1:100	1:200	1:400	Total
Grippotyphosa	26 (35.6%)	3 (4.1%)	0 (0%)	29 (39.7%)
Canicola	14 (19.3%)	6 (8.2%)	2 (2.7%)	22 (30.2%)
Pomona	10 (13.7%)	2 (2.7%)	0 (0%)	12 (16.4%)
Hardjo	4 (5.5%)	2 (2.7%)	0 (0%)	6 (8.2%)
Icterohaemorrhagiae	3 (4.1%)	1 (1.4%)	0 (0%)	4 (5.5%)
Ballum	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	57 (78.2%)	14 (19.1%)	2 (2.7%)	73 (100%)

Table 3: Seropositivity for Leptospirosis in the slaughtered sheep according to the season of sampling

Season	No. of tested	No. of positive (f)	Prevalence (%)
Winter	110	8	7
spring	89	19	21
Summer	80	9	11
autumn	80	30	37.5
Total	359	66	18.4

Statistical analysis: $0.001<p<0.005$

Table 4: The results of statistical analysis of Seropositivity for Leptospirosis at each season as compared with other ones

Comparison of seasons	Statistical analysis
Winter-Spring	$0.005<p<0.01$
Winter-Summer	$p>0.05$
Winter-Autumn	$0.001<p<0.005$
Spring-Summer	$0.01<p<0.05$
Spring-Autumn	$0.01<p<0.05$
Summer-Autumn	$0.001<p<0.005$

Table 5: Seropositivity for Leptospirosis in the slaughtered sheep according to age and sex

Variable	No. of tested	No. of positive (f)	Prevalence (%)	p-value
Age (pairs permanent teeth)				
0	77	6	8	
1	112	9	8	
2	81	15	18	
3	43	21	48	
4	46	15	32	
Total	359	66	18.4	$0.001<p<0.005$
Sex				
Male	296	47	16	
Female	63	19	30	
Total	359	66	18.4	$0.01<p<0.05$

DISCUSSION

Leptospirosis is an infectious zoonotic disease and infections with different serotypes of the leptospires in any geographical area are important on the epidemiology and pathogenesis of the disease in the region. Cattle are maintenance host for many serotypes of the bacteria, thus previous studies on cattle have demonstrated relatively high prevalence rates of the infection in different country and even various regions in Iran. Seroprevalences of leptospiral infections in cattle of Tehran suburb dairy farms at 1990 and 2001 were 31.2% (Moharamie, 1990) and 46.8% (Golie, 2001) and in East Azarbaijan province at 1993 and 2007 were 48.5% (Shoaei, 1993) and 24% (Hasanpour *et al*., 2007), respectively.

Sheep are not naturally maintenance hosts for some of the serotypes such as *Pomona* or *hardjo* and are likely to have infections of relatively short duration, producing severe pathologic effect. However, persistent leptospiruria and high seroprevalence rates of the infection in sheep where no contact with cattle have occurred suggest that sheep may be a maintenance host for some serovars. This could complicate control of the infection in cattle and sheep and infected sheep are a potential zoonotic risk to humans such as abattoir workers, sheep farmers and shearers which previously had not been considered (Radostitis *et al*., 2007).

We found that the seroprevalence of leptospiral infection in sheep in Tabriz was 18.4%. The reported results of seroprevalence of leptospiral infection in sheep are different from region to region or country to country. These differences may be the consequence of environmental factors and control efforts. The environmental factors have been shown to have influential efforts on development of leptospiral infection in animal and human beings. Long-term survival of pathogenic leptospires outside the host requires a warm, moist environment with a near natural PH (Miller *et al*., 1991). So that seroprevalence of leptospiral infection in sheep has been reported to be 60.4% in India (Sratname *et al*., 1992), 19.7% in Argentina (Draghi *et al*., 1984), 16.8% in Greece (Burriel *et al*., 2003), 14.3% in Bolivia (Ciceroni *et al*., 1997), 6.1 and 12.3% in Italy (Ciceroni *et al*., 2000; Cerri *et al*., 2003), 42% in Australia (Ellis *et al*., 1994), 40% in Belize (Everard *et al*., 1988) and 32% in Croix (Ahl *et al*., 1992). The results of this study showed that the serological infection rate in sheep in Tabriz is relatively high and consequently the preventive methods must be applied to prevention of the spread of disease and its transmission to the human and other farm animals.

In comparison to previous studies in Tabriz, the prevalence of antibodies to one or more serovars of L.interrogans was 48.5% (Shoaei, 1993) and 24% (Hasanpour *et al*., 2007) in cattle. Although, the significance of these differences was not defined, but it may be due to differences in susceptibility of these animals. Leptospirosis occurs in sheep and goats with less frequency than in cattle. So that the prevalence of leptospiral infection in cattle, buffalo and sheep in Egypt was 34.5, 26.1 and 4.2%, respectively (Maronopot and Barsoum, 1992). In Turkey, 44.77% of cattle and 8% of sheep reacted to one or more serovar of *L. interrogans* (Ozdemir and Erol, 2002). In Malaysia 40.5, 31 and 10% of cattle, buffalo and sheep reacted to one or more serovar of *L. interrogans*, respectively (Bahaman *et al*., 1987). In the present study, like some of the other ones (Sratname *et al*., 1992; Ahl *et al*., 1992; Ellis *et al*., 1994; Everard *et al*., 1988) seroprevalence rate of leptospiral infection in sheep was relatively high, which emphasize the important role of sheep on the epidemiology of the infection.

In this study *grippotyphosa* and *canicola* were detected as the most prevalent serovars with 39.7 and 30.2%, respectively. With attention to the fact that the rodents and dogs are the major maintenance hosts for *grippotyphosa* and *canicola* serovars, respectively (Radostitis *et al*., 2007) and considering that frequent contacts between sheep and these animals in the flocks of the region, the relatively high prevalence of theses serovars in this study are justified. Thus the preventive methods must be applied to control of the infection in rodent and accompanied flocks dogs.

On the other hand, in previous studies in Tabriz and Ahvaz, the predominant serovars in cattle were *pomona, grippotyphosa* (Hasanpour *et al*., 2007) and *Pomona* (Haji Hajikolaei *et al*., 2007), respectively . It is probable that these serovars may be adapted to and maintained by these farm animals in Tabriz. There is a need for further investigation on clinical cases of leptospirosis to determine whether this serovar is the main cause of leptospirosis in this area. The predominant leptospira serovars in serological reaction varies somewhat from country to country. For example, *poi* and *pomona* in Bolivia (Ciceroni *et al*., 1997), *wollfi*, *pomona* and *ballum* in Argentina (Draghi *et al*., 1984), *hebdomadis* in the UK (Hathaway *et al*., 1981), *pomona* in India (Manickavel *et al*.,1991), *autumnalis* in Egypt (Maronpot and Barsoum, 1992), *castellonis* in Italy (Ciceroni *et al*., 2000), bratislava in Greece (Burriel *et al*., 2003), canicola in Portugal (Levett, *et al*., 1996) icterohaemorrhagiae, *pomona* in south America (Saglam *et al*., 2007) and *pomona* in Malaysia (Bahaman *et al*., 1987) were the predominant serovars in sheep. In addition, one serovar may be predominant in a country but none of the animal reacted with this serovar in another country. This emphasizes the need for regional surveys for leptospirosis, since host-parasite relationship may change depending on the ecology of the region.

Antibodies against more than one serovar were found in 10.6% of seropositive sheep. In serologic tests for leptospirosis such as MAT, the results often indicate infection with more than one serovar (Hathaway *et al*., 1981; Firouzi and Vandyousefi, 2000). This may be the result of mixed serovar infection or cross-reactivity among serovars.

The high prevalence of infection and dominant titre of 1:100 reveal that leptospiral infection in sheep in Tabriz is endemic and occurs mostly in subclinical form.

Statistical analysis of the results showed that the rates of the infection in the autumn (37.5%) and spring (21%) were significantly higher than the other seasons ($0.001<p<0.005$). Large amounts of raining and transferring the sheep flocks in theses seasons can increase the infection rate of leptospirosis (Howard and Smith, 1999). Although in different studies, most prevalence rate of the infection occurs in varies seasons depend on the regions and serovars. For example, *hardjo* and *pomona* were the most common detected serovars, in highly raining and dried season or areas, respectively, in Spain (Howard and Smith, 1999). These differences may be the consequence of diversity of weather and environmental factors in various regions, so that in highly raining, warm and moist environments the infection is prevalent in all seasons (Miller *et al*., 1991; Rocha, 1998).

The rate of the infection has been statistically increased with the aging ($0.001<p<0.005$) and the animals with three and four pair's permanent teeth (4-5 years old) had the highest infection rates. The finding is similar to the results of the other surveys. Overall the prevalence rate is increasing with aging the animals due to enhancing probability of contact of animals with organism (Ciceroni *et al*., 2000; Saglam, *et al*., 2007).

CONCLUSION

In overall conclusion, it seems that the serological infection rate in sheep in Tabriz is relatively high and consequently the preventive methods must be applied to prevention of the spread of disease and its transmission to the human and other farm animals.

ACKNOWLEDGEMENT

The authors would like to thank the Research Vice Chancellors of Islamic Azad University of Tabriz for financial support of this project.

REFERENCES

Ahl, A.S., D.A. Miller and P.C., Bartlett, 1992. Leptospira serology in small ruminants on St. Croix, U.S. Virgin Islands. Ann. N.Y. Acad. Sci., 653: 168-171.

Bahaman, A.R., A.L. Ibrahim and H. Adam, 1987. Serological prevalence of leptospiral infection in domestic animals in west Malaysia. Epidemiol. Infect., 99: 379-392.

Bulu, A.A., R. Dorterler, O. Ozkan and F. Hasturk, 1990. The studies on the spreading and the serotype of leptospirosis occurrence in cattle and sheep in some cities of the East Anatolia. J. Etlik Vet. Microbiol., 6: 49-60.

Burriel, A.R., C. Dalley and M.J. Woodward, 2003. Prevalence of leptospira species among farmed and domestic animal in Greece. Vet. Rec., 153 (5): 146-148.

Carter, G.R. and M.M. Chengappa, 1991. Essential of Veterinary Bacteriology and Mycology. 4th Edn. Lea and Febiger, pp: 220-223.

Cerri, D., V. Ebani, A. Pedrini, E. Andreani, R. Farina and P. Pinzauti, 2003. Epidemiology of leptospirosis: Observations on serological data obtained by a diagnostic laboratory for leptospirosis from 1995 to 2001, New Microbiol., 26 (4): 383-389.

Ciceroni, L., A. Bartoloni, A. Pinto, P. Guglielmetti, C. Valdez Vasquez, H. Gamboa Barahona, M. Roselli, F. Giannico and F. Paradisi, 1997. Serological survey of leptospiral infections in sheep, goats and dogs in Cordillera province, Bolivia. New Microbiol., 20 (1): 77-81.

Ciceroni, L., D. Lombardo, A. Pinto, S. Ciarrocchi and J. Simeoni, 2000. Prevalence of antibodies to Leptospira serovars in sheep and goats in Alto Adige-South Tyrol. J. Vet. Med., 47 (3): 217-223.

Draghi de Benitez, M.G., M.A. Zubriggen and V.R. Vanzini, 1984. Serological survey for ovine leptospirosis in Correentes province. Argentina. Vet. Argent., 1: 336-340.

Ellis, G.R., D.L. Partington, M. Hindmarsh and M.D. Barton, 1994. Seroprevalence to Leptospira interrogans serovar hardjo in merino stud rams in South Australia, Australian Vet. J., 71 (7): 203-206.

Everard, C.O.R., F. Cawich, P.G. Gamble and J.D. Everard, 1988. Prevalence of leptospirosis in Belize, Transactions Royal Soc. Trop. Med. Hygiene., 82 (3): 495-499.

Firouzi, R. and J. Vandyousefi, 2000. A serological survey on bovine leptospirosis in Shiraz. Iranian J. Vet. Res., 2: 118-123.

Golie, G., 2001. Seroepidemiologic study on leptospiral infection in cattle in Karaj. Thesis for graduation in DVM Degree, University of Tehran, No of thesis: 17T.

Hajikolaei, M.R., M. Ghorbanpour, D. Gharibi and G.R. Abdollapour, 2007. Serologic study on leptospiral infection in sheep in Ahvaz, southwestern Iran. Iranian J. Vet. Res., University of Shiraz, 8 (4) 21: 333-336.

Hasanpour, A., M. Fartashvand, G.R. Abdollapour, G. Mogadam, M.G. Nadalian and S. Sattari, 2007. Seroprevalence of leptospiral infection in dairy herds in Tabriz- Iran. J. Res. Reconstruct., 74: 67-77.

Hathaway, S.C., T.W.A. Little, S.M. Finch and A.E. Stevens, 1981. Leptospiral infection in horses in England: A serological study. Vet. Rec., 2: 396-398.

Howard, J.L. and Smith, R.A., 1999, Current Veterinary Therapy, Food Animal Practice. 4th Edn. W.B. Saunders, pp: 352-357, 637.

Levett, P.N., C.U. Whitington and E. Camus, 1996. Serological survey of leptospirosis in livestock animals in the Lesser Antilles. Ann. N.Y. Academy Sci., 791: 369-377.

Manickavel, K., C.K. Kalyanasundaram, K.S. Venkat-araman, V.N.A. Rao and S. Thanagavelu, 1991. Reports on leptospirosis in sheep in Tami Nadu. Indian Vet. J., 68: 503-505.

Maronopot, R.R. and I.S. Barsoum, 1992. Leptospiral microscopic agglutination antibodies in sera of man and domestic animals in Egypt. Am. J. Trop. Clin. Microbiol., 30: 2219-2224.

Miller, D.A., M.A. Wilson and G.W. Beran, 1991. Relationship between prevalence of Leptospira interrogans in cattle and regional climate and seasonal factors. Am. J. Vet. Res., 52: 1761-1768.

Moharamie, M., 1990. Seroepidemiologic study on leptospiral infection in cattle in Tehran. Thesis for graduation in DVM Degree, University of Tehran, No of thesis: 1928.

OIE, 2000. Manual of standards diagnostic tests and vaccines, leptospirosis, Paris. Part 2, Section 2.2, Chapter 2.2.4. http://www.OIE.int.

Ozdemir, V. and E. Erol, 2002. Leptospirosis in Turkey. Vet. Rec., 150: 248-249.

Quinn, P.J., B.K. Markey, M.E. Carter, W.J. Donnelly and F.C. Leonard, 2002. Veterinary Microbiology and Microbial Disease. Blackwell, pp: 175-184, 453-455, 484.

Radostitis, O.M., C.C. Gay, K.H. Hinchcliff and P.D. Constable, 2007. Veterinary Medicine. 10th Edn. Saunders Elsevier, London, pp: 971-985.

Rocha, T., 1998. A review of leptospirosis in farm animals in Portugal. Revue Scientifique Et Technique (International Office Of Epizootics), 17 (3): 699-712.

Saglam, Y.S., Z. Yener, A.Temur and E. Yalcin, 2007. Immunohistochemical detection of leptospiral antigens in cases of naturally occurring abortion in sheep. Small Ruminant Research (in Press).

Shoaei, S., 1993. Serologic study on leptospiral infection in cattle in East Azarbaijan provience. Thesis for graduation in DVM Degree, Azad University of Tabriz, No of thesis: 20.

Sratname, K.L., A. Coreverad, M.I. Bsuresh and J.C. Alex, 1992. Leptospiral antibodies among sheep and goats. Indian J. Anim. Sci., 62: 1041-1043.