Article

PROBABILITY OF EXPOSURE TO AGENTS OF TICK – BORNE ZOONOSES IN AOSTA VALLEY

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Abstract: We estimated the probability of exposure of people to tick – borne bacterial agents in Aosta Valley, western Alps, Italy. We collected questing ticks by dragging a 1 m^2 cloth on 100 m of land, in three hiking trails, which were divided into an internal path, with low vegetation, and into an edge with higher grass. Ticks were also collected from people clothes. *Borrelia burgdorferi* s.l. was identified by PCR in 40.0% (95% CI = 22.5, 57.5) of *Ixodes ricinus* (L.) nymphs. Prevalence of infection by *Rickettsia* spp. was 13.3% (95% CI = 1.2, 25.5). The probability of encountering at least one host-seeking *I. ricinus* infected by each bacterial agent (probability of exposure, *E*) in 100 m^2 was obtained by combining the number of collected nymphs, prevalence of infection by each bacterial agent, frequency of passage by visitors, and the probability of tick attachment to people. The mean number of nymphs collected by dragging was greatest in the internal part of hiking trails (mean = 7.9), *E* was greater on the external edge (up to 0.14 for *B. burgdorferi sl*, and 0.07 for *Rickettsia* spp.), due to a greater probability of tick attachment to people in higher vegetation.

Keywords: Risk analysis; *Ixodes ricinus*; *Borrelia burgdorferi s.l.*; *Rickettsia* spp.; ticks; zoonoses; Italy

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1. Introduction

I. ricinus is a hard tickthat is able to transmit a wide range of pathogens, such as viruses, bacteria, and protozoa, which can cause illnesses in both animals and humans [1]. Among bacteria, *Borrelia burgdorferi sensu lato* (*s.l.*) and *Rickettsia* spp. are frequently detected in Europe [2]. *I. ricinus* and transmitted agents are widely distributed, from southern Spain to northern Scandinavia [3]and, in recent decades, they have been repeatedly reported from the Alps, at altitudes above 1000 – 1200 m above the sea level (a.s.l.), which were previously considered as the maximum altitudinal limit of the tick's

geographic range [4]–[6]. Ticks and tick – borne zoonoses have been more frequently studied in the northeastern, Italian Alps [7]–[9], nevertheless recent studies have highlighted their presence also in North western Italian regions [5], [10]. Accordingly, tick bites have been increasingly reported by Parini Hospital Service in Aosta Valley (personal communication [11]). Since we are referring to a geographical residential area, where occupational and recreational activities expose people to tick bites and, consequently, to their transmissible pathogens, we decided to assess this probability of exposure. To reach this aim, we applied a risk assessment approach, following OIE terminology adapted for zoonoses' investigations [12]. In general, the risk assessment consists of three main parts: (1) *hazard characterization*, (2) *release assessment* and (3) *exposure assessment*. In this contest, we intended (1) as the study of a biological hazard, namely ticks and some tick-borne diseases (TBD) frequently identified in alpine areas; (2) as the quantification of ticks and transmitted pathogens "released" by the environment of the selected study area; (3) as the probability of people's contact with the specific biological hazard.

This approach is a tool intended to be adaptable to different contests and geographical territories, useful to compare risk levels and to direct preventive and control measures consciously.

2. Materials and Methods

2.1. Hazard characterization

2.1.1. Ticks collection

Ticks were collected from May to July 2016 in a municipality located in Aosta Valley, North West of Italy (45°47′N 7°19′E), where human tick bites had been reported. Three different hiking trails (A, B and C) were selected, considering variability in altitude and vegetation cover. The trails were located in an area ranging from 780 to 1140 m a.s.l., mostly characterized by downy oak (*Quercus pubescens*), except for trail C where scots pine (*Pinus sylvestris*) was the predominant tree species. Each trail was divided in transects of 100 m^2 on which we performed several dragging sessions, stopping at each 25 m^2 to check the attachment of ticks on the white drag, and on the operators' clothes. Every transect was split in an internal part (low vegetation) and in an external part (high vegetation) where we collected host-seeking ticks separately. During two days of sampling, a white short hair dog was allowed to walk freely along the path with the operators and ticks were collected from its hair. Nevertheless, in this case it was impossible to distinguish between internal/external part.

Before performing the sampling, a data sheet was filled with GPS coordinates (UTM system), temperature and humidity at each transect, by using a smartphone and a HI 8564 thermo hygrometer (Hanna Instrument Italia, Milano).

We decided to investigate only nymphs and adults' presence and infection, since larvae are the less risky stadium for diseases' transmission. Collected ticks were preserved

in 70% ethanol and subsequently identified under a microscope using taxonomic keys by Manilla (1998) [13].

2.1.2. Molecular analysis

A random sample of 30 *I. ricinus* nymphs was screened by PCR to detect *B. burgdorferi s.l.* and *Rickettsia* spp. as described Tomassone et al. (2017) [14]. For the DNA extraction we used DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany 50). To target *B. burgdorferi* s.l. an intergenic spacer region included between genes coding for the 5S and 23S subunits of ribosomal RNA was amplified. On the other hand, *Rickettsia* spp. infection was investigated using two consecutive PCRs targeting the gltA gene first and then ompA gene, to characterize Spotted Fever group [15]. ~~The PCR run was performed at 130 V for 50 min on a gel produced with 3 g of agarose and 150 ml of TAE buffer.~~ Amplicons were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Chalfont, UK) and sent to an external service for sequencing (BNR Genomics, Padova, Italy). Sequences were analyzed and submitted to BLAST (www.ncbi.nlm.nih.gov/BLAST) for comparison to known sequences.

2.2. Release assessment

To assess the release of infected ticks by the environmental source, we estimated the probability of collecting at least one infected tick by dragging on a 100 m transect, by using the following equation [16]:

(1)

The equation derives from the combination of two factors: the density of ticks (*DT*) and their prevalence of infection (*p*). The value of *p* was obtained from the PCRs results as the proportion of nymphs positive to *B. burgdorferi s.l.* and *Rickettsia* spp. out of the total, combining data from all of the three hiking-trails, given the relatively small sample of tested ticks. On the other hand, *DT* was calculated as the mean number of nymphs per 100 m^2 dragging, first considering the three hiking-trails separately and, subsequently, discriminating between internal and external part. Therefore, *DT* that could be differentiated for path (A, B and C) and subpart of it (internal/external).

2.3. Exposure assessment

2.3.1. Probability of exposure (E)

To provide a more realistic data of the ticks' attachment probability we decided to adapt Verheyen and Ruyts (2016) equation [17], slightly modified. As a matter of fact, we decided to express the equation as the probability of exposure (E) to generalize the concept, since we didn't investigate just *B. burgdorferi* infected ticks, but also *Rickettsia* spp. The probability of exposure or E can be defined as the probability of a person making contact with at least one infected tick along a 100 m forest trail. The equation combines three main factors: visitor flow (v), contact probability with questing nymphs (c) and the release (R).

(2)

The presence of infected ticks in a specific spatio – temporal context (R) depends upon the agents' transmission dynamics in the geographic area, whereas the probability of being bitten (exposure) by an infected tick depends upon activities leading to human frequentation (v) and tick attachment to people (c) in the considered area. For clarity's sake, R and *dragging* can point the area with a higher concentration of infected nymphs, but if this area is the less frequented then the resulting exposure (E) could be limited.

The parameter E has been estimated for the three hiking trails (A, B and C) separately and divided in internal and external part.

We considered factor v as the probability of at least one visitor per hour, based on the records noted during the sampling sessions, using the following formula:

(3)

[3]: "VH" stands for the number of visitors per hour. For example, in trail C we supposed a passage of 1 visitor every 4 hours, so "VH" corresponded to 0,25.

On the other hand, factor c, considered as the probability of contact between the visitor and questing nymphs, has been calculated as the proportion of the mean number of nymphs collected by *walking* out of the ones collected by *dragging*. This factor should estimate the probability of attachment of questing nymphs "released" in the study area.

2.3.2. Questionnaire

To integrate information on the exposure of people to questing ticks in the examined trails, we administered a short questionnaire to residents in the study area. The following questions were included: 1) number of people in the household; 2) number of people carrying out working or recreational activities in the specific hiking trails; 3) occurrence of tick bites on components of the households; 4) geographic location of tick bites, to be identified on a municipality map. The questionnaires (n = 355) were manually delivered into mailboxes of each house of the municipality, asking to return the filled questionnaires in a box in the city hall.

3. Results

3.1. Hazard characterization

We carried out 42 collections, 18 on internal part (low vegetation) and 18 on the external one (high vegetation). Dragging positivities of 83% and 78% have been identified for the external part and for the internal one, respectively. This prevalence means that on 100 collections 83 showed the presence of at least one tick on the cloth dragged on the external part and 78 on the internal one.

A total of 347 ticks were collected; 345 were microscopically identified as *I. ricinus* and 2 as *Dermacentor marginatus*. Questing ticks (n=316) were collected by dragging in 9 transects (3 of trail A; 4 for trail B; 2 for trail C): 285 nymphs and 31 adults of *I. ricinus* and 2 adults of *D. marginatus*. The questing *I. ricinus* collected by walking were 20 in total, 11 nymphs and 9 adults. A total number of 9 *I. ricinus* adults were found on the coat of the dog that accompanied the operators during two collecting sessions.

The mean number of nymphs collected by dragging in 100 m of transect was n=7.9 for the internal part and n=4.4 for the external one. Considering the three paths separately the mean number was n=12.5 for path A, n=4.75 for path B and n=3.5 for path C. In this study the mean number of nymphs has been intended as the density of ticks (*DT*) used in the release (*R*) calculations. Adult stadium was not considered in the following mathematical calculations, but to be complete we can just assert that the proportion between adults collected by dragging in the internal (n= 0.44) and external (n=0.94) part is inversed in comparison with nymphs.

From PCRs of 30 randomly chosen *I. ricinus* nymphs, it resulted a prevalence (*p*) of 40% (12 positives; IC 95%: 22.5-57.5) for *B. burgdorferi s.l.* and 13.3% (4 positives; IC 95%: 1.17-25.50) for *Rickettsia* spp. Sequence analysis highlighted the presence of *B. afzelii* (p= 33.3%; 10 positives; IC 95%: 16.5-50.2) and *B. valaisiana* (p= 3.3%; 1 positive; IC 95%: 0-9.8). Only 1 of the 4 *Rickettsia* spp. positive could be identified as *R. helvetica*.

3.2. Release assessment

To use the release equation (*R*) we evaluated the prevalence of infection (*p*) and the density of ticks (*DT*), resulting from the *hazard characterization*. The calculation of *R*

showed a higher probability of encountering an infected nymph in the internal part of the 100 m of all paths and, particularly, in trail A.

The results are graphically represented in Figures 1 and 2.

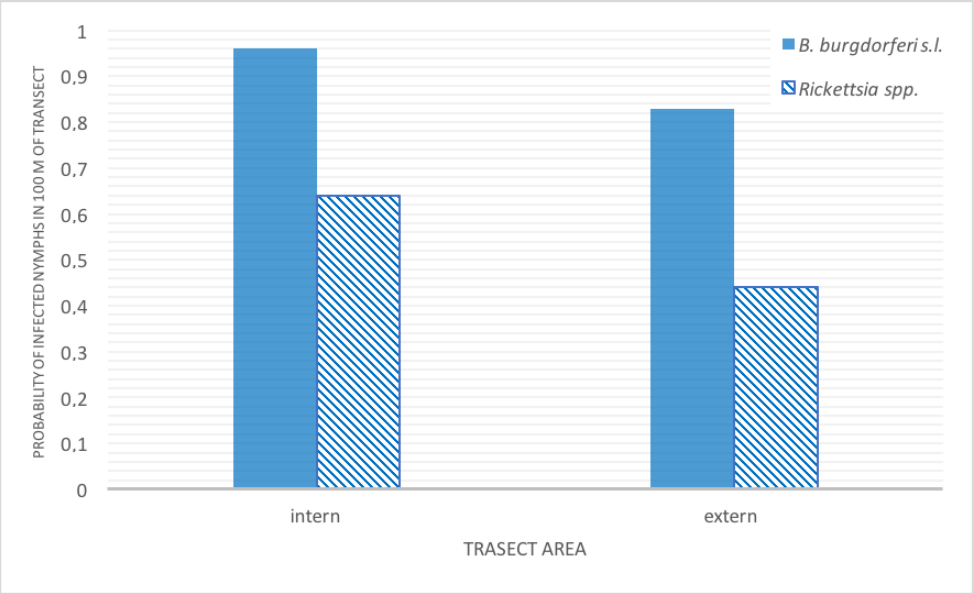

Figure 1. Results of the release (*R*) calculations of *B. burgdorferi s.l.* and *Rickettsia* spp. of the internal part (intern) and the external one (extern), considered separately.

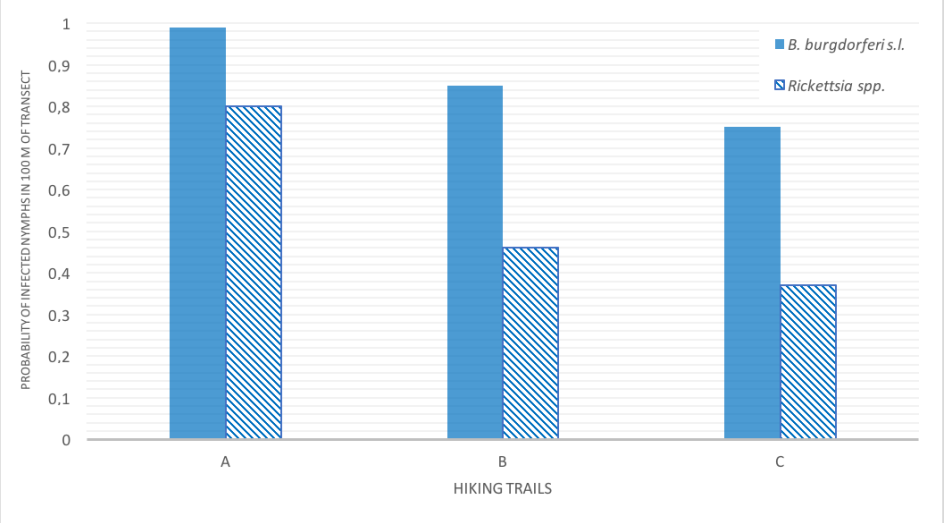

Figure 2. Results of the release (*R*) calculations of *B. burgdorferi s.l.* and *Rickettsia* spp. in the three selected trails (A, B and C), considered separately.

3.3. Exposure assessment

The calculation of *E* has shown that the risk of exposure to infected nymphs is irrelevant in the internal area (*E*=0) because no ticks have been collected from walking (*c*=0). On the contrary, in the external part there is a certain probability to enter in contact with infected questing nymphs. Therefore, the calculation of *E* showed a higher probability of exposure to an infected nymph in the external part of the 100 m of all paths and, particularly, in trail B.

Results are listed in Table 1 and graphically represented in Figure 3.

Table 1. Factors and results of the probability of exposure (*E*) "$Mn_{walking}$" mean number of nymphs collected from operators' clothes; "$Mn_{dragging}$" mean number of nymphs collected from the drag; "c" ratio between $Mn_{walking}$ and $Mn_{dragging}$; "Passages" rate of time in which one visitor is seen; "VH" number of visitors per each hour; "v" probability of at least one visitor per hour (see [3]); "R Bb" release of *B. burgdorferi s.l.* (see [1]); "R Rick" release of *Rickettsia* spp. (see [1]); "E Bb" probability of exposure to *B. burgdorferi s.l.* (see [2]); "E Rick" probability of exposure to *Rickettsia* spp. (see [2]).

Trail (external part)	Calculation of *c*			Calculation of *v*			*R*[3]		*E*[4]	
	$Mn_{walking}$	$Mn_{dragging}$	*c*[1]	Passages	VH	*v*[2]	Bb	Rick	Bb	Rick
A	0.75	10.5	0.07	1 in 2h	0.5	0.39	0.99	0.80	0.035	0.028
B	0.2	2.6	0.08	1 in 30′	2	0.86	0.85	0.46	0.136	0.074
C	0.25	3	0.08	1 in 4h	0.25	0.22	0.75	0.37	0.015	0.007

[1] $c = Mn_{walking} \div Mn_{dragging}$; [2] $v = $; [3] $R = $; [4] $E = v \times c \times R$

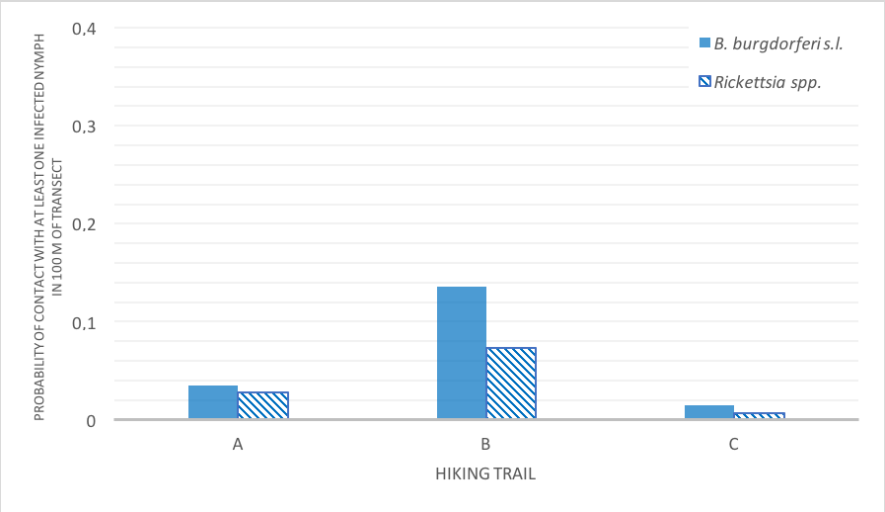

Figure 3. Results of the probability of exposure (*E*) to *B. burgdorferi s.l.* and *Rickettsia* spp. in the three selected trails (A, B and C), considered separately.

We received back 60 of the 355 delivered questionnaires. Based upon results, the mean number of people per household was 2.7, and 89.1% of household members use the hiking

trails for recreational reasons. Tick bites were reported on 46.8% of people carrying out recreational activities. In ten out of 20 questionnaires including map locations, tick bites occurred on trail B, whereas only one bite was reported in trail A, and none in trail C. The rest of bite locations were outside of the studied trails.

4. Discussion

This study aimed fist to identify and quantify the ticks in a restricted area of Aosta Valley, secondarily to assess the probability of exposure of people to these vectors and their pathogens.

I. ricinus was the most abundant species collected in the three selected hiking trails. Probably, the deciduous forest, the leaf litter, and the wild animals that characterize the study area create a favorable habitat for the completion of the developmental cycle of this tick species. If we consider the density of ticks (*DT*) of the internal part of the paths, it resulted quite important. Indeed, comparing the mean number obtained from our study (*DT*= 7.9 nymphs per 100 m^2 of transect) with bibliographic data referring to neighboring regions (*DT*= 2.6–3.5 nymphs/100 m^2 Piedmont [5]; *DT*= 0.16-0.50 nymphs/100 m^2 Liguria [10]) we can affirm that the density of ticks of the study area is substantially high.

A prevalence of 40% (CI 95%: 22.5-57.5) of *B. burgdorferi s.l.* has been detected. In general, in Italy *B. burgdorferi* s.l. prevalence in ticks is variable, depending on the geographical area examined: it goes from a 10.6% of prevalence in Piedmont to a 40.1% in Trentino Alto Adige [5]. The latter is a region similar to Aosta Valley in many aspects, as the morphology of the territory and the climatic conditions, and it could be possible that these characteristics determine a higher prevalence value. Among the genospecies of *B. burgdorferi s.l.* group, the most represented in the study area were *B. afzelii* and *B. valaisiana*. *B. afzelii* is the principal cause of cutaneous borreliosis and it is maintained in nature by small mammals. Probably, the study area presents an adequate amount of food, a low number of predators and a large vegetation cover, factors that influence the diffusion of the reservoirs on the territory [18]. In future investigations it could be interesting to use trapping as a complementary method to evaluate the species, abundance, level of infestation and infection of small mammals.

Mammals are not the only class that play a role in the maintenance of ticks and tick-borne diseases (TBD), but also birds have to be considered. Especially ground-feeding birds are fitting hosts for larval and nymphal stages of *I. ricinus* and its infections [19]. The other genospecies identified in our study is *B. valaisiana*, a borrelia typically maintained by birds and considered only potentially pathogenic. Birds could be the reservoir of other genospecies, like *B. garinii* the etiological agent of neuroborreliosis. It can not be excluded the presence of the latter pathogen in the remaining untested nymphs, which could be analyzed in future researches.

Regarding *Rickettsia* spp. we found a prevalence of 13.3% (CI 95%: 1.17-25.50), a significant value if we consider an European variable mean range of 3-14% [20]. *R. helvetica*

is the only species identified by DNA sequencing. In the last few years, some cases of rickettsiosis caused by *R. helvetica* have been reported in Europe, which could confirm its pathogenicity in humans [20]. The role of wild animals in the maintenance of *R. helvetica* is still open, even though it has been hypothesized birds should be competent reservoir of the infection, since they are not just carrier of ticks but they develop a bacteraemia [21].

These results are important, especially for the local health service, because they help to define the epidemiological picture of TBD on the territory and to direct the correct diagnosis after a tick bite. We tried to evaluate the risk of people frequenting the three hiking trails where ticks have been collected. As previously mentioned, the internal part of all paths, and in particular trail A, showed a higher probability of encountering infected nymphs (*R*). The concentration of nymphs in the internal part could be due to the method of collection chosen, that influence factor *DT*: *dragging* is very sensitive for the collection of nymphs in low vegetation considering their questing behaviour. In fact, nymphs wait for the host on vegetation's lower levels in comparison with adults, principally because they feed on medium-size animals and they are more subjected to drying [22]. For that reason, it could be possible that the drag is unable to penetrate accurately in the deepest part of external stems to permit the contact between the nymphs and the cloth. On the contrary, the internal part, characterized by low vegetation, facilitates the attachment of this tick growth stage. Our results are in line with the previous statements since nymphs and adults have been collected more frequently in the internal (low vegetation) and external (high vegetation) parts, respectively. The high value of *R* obtained on trail A could depend on biotic and abiotic influencing factors, such as abundance of hosts in the area and habitat conditions (type of vegetation, temperature and humidity) more suitable for nymphs' development and maintenance [23], [24]. Indeed, trail A is characterized by a lower mean temperature ($A_{T°}$=23.1 °C) and a higher mean humidity (A_{RH}=70.5%) compared to trails B and C ($B_{T°}$=26.3 °C; B_{RH}=63.8%; $C_{T°}$=30.6 °C; C_{RH}=54.4%). Moreover, in trail A there is a concentration of downy oaks (*Quercus pubescens*), which fruits can be consumed by some nymphs' hosts, like wild rodents.

Nevertheless, the high *R* identified in trail A doesn't necessary reflect an equivalent probability of exposure *E* to tick-borne pathogens. The release *R* just suggests the infestation level of a specific area; in other words, how the ecosystem "produces" a larger amount of infected nymphs, which maybe will never enter in contact with humans and transmit the agents. Consequently, we supposed that calculating *E* could provide a more realistic overview of the exposure to tick-borne pathogens.

Considering the results shown in Table 1, we can affirm that, for example, walking for 1 hour on 100 m length of trail B's external part the probability of being exposed to at least one nymph infected with *B. burgdorferi s.l.* and *Rickettsia* spp. is 13.6 % and 7.4 %, respectively. In literature there are not applied studies to which we could compare our results. However, this mathematical model has been used theoretically by Verheyen and Ruyts (2016) [17] assuming *c*=1.0 if the vegetation is higher than 50 cm and c=0.1 if it's lower than 50 cm. Therefore, we tried to use *c*=1 for the external part (high vegetation) and

c=0.1 for the internal part (low vegetation). In this case, the probability of exposure (*E*) to infected nymphs in the internal part results > 0%, even if the value is still low. The other values of the external part result similar to the ones calculated with the factor *c* obtained from data collected on field. So, we can affirm that Verheyen and Ruyts' theoretical approach is an approximation that could be used when there is a loss of information from *walking*.

In both formulations, data show that remaining in the internal part and avoiding the contact with the high vegetation, can reduce the probability of exposure to ticks and tick-borne diseases.

If we compare the result of *R* and *E* we can see that there is not a direct proportion between the two models. In fact, if trail A is the path with higher value of *R*, trail B is the one with the highest *E*. For that reason, *R* gives an overview on the most infested areas, but the real risk for people depends on human's contact with the vegetation (*c*) and how frequently are visited these areas (*v*). Therefore, to assess a more realistic probability of exposure, it could be useful to combine the above-mentioned factors. Results of the questionnaire on trail use and tick bites on people are in agreement with our estimate of a greatest *E* on trail B, although frequency of human activities on each trail was not included in the survey. On the other hand, filled questionnaires comprised information on other locations where tick bites were recorded, and which might be included in further studies.

5. Conclusions

In Aosta Valley the probability to be exposed to agents of TBD exists, certainly to *B. burgdorferi s.l.* and *Rickettsia* spp., which are transmitted by *I. ricinus*. Precisely, walking in the internal part of hiking trails seems to be a preventive behavior to reduce exposure to TBD, even if greatest number of nymphs can be collected by *dragging* on this trail section. This apparent contradiction could be due to the limitations of *dragging* as a method to collect nymphs on high vegetation. For that reason, we suggest to combine data obtained from both *dragging* and *walking* methods by calculating factor *c* (contact probability with questing nymphs) and to use the probability of exposure model (*E*), in TBD risk assessment. It could be useful to improve the evaluation of factor *v* (visitor flow) by observing the passages of people in each trail for more hours, to have a more realistic mean number of visitors per hour. Finally, our pilot field study could be extended to other trails and geographic areas of the region, to build a risk map, and to direct control, prevention and communication of the probability of exposure to agents of TBD.

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Conflicts of Interest: The authors declare no conflict of interest.

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