ORIGINAL ARTICLE



The role of *Linguatula serrata* nymph in transmission of enteric bacterial pathogens to internal organs in sheep

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Abstract Linguatula serrata is a worldwide zoonotic parasite belong to phylum Athropoda. When the eggs are swallowed by intermediate host, the larvae are released in intestine and reach the mesenteric lymph nodes (MLNs) and occasionally liver, lungs, heart, kidneys, spleen, and other body organs by the blood and lymph circulation. There are a few evidences showing transmission of microorganisms by migrating L. serrata. The aim of this study was to determine the role of L. serrata nymph in transmission of enteric bacterial pathogens to internal organs of sheep. For this purpose 11 parasite positive and 11 parasite negative MLNs to L. serrata were obtained from the native slaughtered sheep and were examined microbiologically in terms of bacterial contamination. The average total bacterial count and Escherichia coli count in the parasite positive samples were respectively 6.7 and 3.3 times higher than parasite negative ones (P < 0.05). However no significant differences were found for Salmonella and intestinal enterococci between parasite positive/negative samples. This indicates that L. serrata

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nymphs play as vehicles for bacteria and so contaminate offal. *L. serrata* nymphs transfer some bacterial agents to internal organs and enhance post mortem spoilage of the infected organs. It is also able to transfer some bacterial pathogens to internal organs which could potentially be the etiology of severe infectious or even zoonotic diseases. Especially in some regions where the consumption of raw or semi-cooked lymph nodes and other visceral organs are common.

Keywords *Linguatula serrate* · Inoculative effect · Mesenteric lymph nodes

Introduction

Linguatula serrata is a world wide zoonotic parasite belong to phylum Athropoda. Its adult lives in the upper respiratory tract of canine as final hosts (Oryan et al. 2008). When the eggs are swallowed by herbivore intermediate hosts such as sheep, goats, cattle, etc., the larvae which are released in the intestine of these animals reach the mesenteric lymph nodes (MLNs) and occasionally liver, lungs, heart, kidneys and spleen. After six to nine molting stages, the larva develops into an encysted infective nymph which is about 500 µm in length. When the infective nymph is swallowed by the final host, it migrates to the upper gastrointestinal tract, and finally localizes in the nasal cavity and throat (Tavasoli et al. 2007; Akhondzadeh Basti and Hajimohamadi 2010; Alborzi and Derakhshandeh 2008; Hajimohammadi et al. 2012; Akhondzadeh Basti et al. 2011).

Several studies have reported high *L. serrata* infection prevalence in slaughtered animals of Iran (Alborzi and Derakhshandeh 2008; Nourollahi Fard et al. 2010a, 2010b;

Hami et al. 2009; Nourollahi Fard et al. 2010a, 2010b; Tavassoli et al. 2007; Rezaei et al. 2012; Shekarforoush et al. 2004; Oryan et al. 2011; Bamorovat et al. 2014; Oryan et al. 1993). Although L. serrata has not been considered as an epidemic parasite in Europe and North America (AkhondzadehBasti and Hajimohamadi 2010), it is endemic in Africa, Asia and most parts of the Middle East especially of Iran. Several studies have reported high prevalence of this parasite in slaughtered animals such as cattle (44%; Tajik et al. 2006), buffalo (16.6%; Tajik et al. 2008), goats (30.6%; SadeghiDehkordi et al. 2013), sheep (65.79%; Yakhchali et al. 2009) and camels (13.5%; Haddadzadeh et al. 2010). It has been stated that traditional grazing of the livestock together with the close contact of the herbivorous intermediate hosts with dogs as the final hosts and, in most instances, accessibility of stray dogs to the infected offal of the intermediate animals result in high prevalence of *L. serrata* in the country (Oryan et al. 2008; Meshgi and Asgarian 2003; Rezaei et al. 2011).

Consumption of raw or semi-cooked infected internal animal organ as the most common way of human infection may lead to coughing, sneezing, pharyngitis, headache and runny nose which are known as Halzoun syndrome (AkhondzadehBasti and Hajimohamadi 2010). In some cases the parasite may migrate to eyes and leads to visual disturbances (Ocular linguatulosis; Koehsler et al. 2011; Lazo et al. 1999). Unhealthy food habits and some traditional beliefs in native human societies are the main risk factors of this disease. Marrara as a meal in sudan consisting of raw intestines and other viscera of sheep and goats mixed with salt, various spices, lemon and onions which is commonly consumed without cooking as a raw food in Sudan results infection (Alzohairy 2014). In some Middle Eastern countries such as Lebanon, consumption of uncooked or semi-cooked MLNs of slaughtered animal is the most important way of human affection (Khalil and Schacher 1965; Khalil et al. 2012; Yagi et al. 1996). In some areas, the consumers of meat products, according to their preference and taste, may not apply enough heat in cooking the grilled liver or other organs, and this may result in infection of humans. These are the most important risk factors listed in the reports of human cases, especially in Iran. On the other hand, consumption of raw or semicooked liver by pregnant women is common in some parts of Iran, because they believe that such diet improves the growth and development of fetus (AkhondzadehBasti and Hajimohamadi 2010). It should be highlighted that one of the most important risk factors listed in the reports of human cases, especially in Iran is attributed to the consumption of raw or undercooked liver. It has also has been observed that Iranian patients with chronic anemia consume raw liver that could be a risk factor in transfer of infection (AkhondzadehBasti and Hajimohamadi 2010; Maleky 2001; Sajjadi et al. 1998; Siavoshi et al. 2002; Yeghaneh Moghadam et al. 2001).

There are a little evidences showing transmission of bacterial microorganisms so called inocolative effect by migrating *L. serrata* from intestine to other organ (Miclăuş et al. 2008; Mir et al. 2009). Therefore, transfer of hazardous intestinal pathogens such as *Salmonella* spp. and *Escherichia coli* to edible organs such as liver and lungs is another risk factor of *L. serrata* hence, the parasite may have a role in transmission of non-pathogenic spoilage-causing bacteria that could result in spoilage of edible offal such as liver.

Therefore, we decided to evaluate the role of *L. serrata* nymph in transmission of intestinal bacterial infection to internal organs in sheep, using the bacterial culturing and histopathological methods.

Materials and methods

Sample collection

This study was a descriptive study. Eleven parasite positive MLNs (with *L. serrate*) and 11 parasite negative MLNs (Fig. 1) were obtained from the slaughtered sheep in an industrial slaughterhouse in Yazd, central Iran. The samples were quickly transferred to the laboratory under aseptic and cold condition $(4 \pm 1 \,^{\circ}C)$. The adipose tissue covering of the lymph nodes were dissected and removed. Then the surface of nodes were sterilized with ethanol and each lymph node were divided into pieces with sterile scalpel and forceps.

Bacterial examination

The samples were diluted (1:9) in sterile Ringer's solution (Merck 1155250001, Darmstadt, Germany) in sterile bags and dumped in stomacher for 5 min to be homogenized. The initial suspension was used for total bacterial count,



Fig. 1 Mesenteric lymph node infected with the nymph of L. serrata

detection and enumeration of *E. coli* and fecal *Enterococci* in each sample. To detect *Salmonella* spp., a 25 g sample was diluted in 225 ml sterile peptone water (Titan Biotech M028, India).

Most probable number (MPN) method (ISIRI 2946 2005; ISO 7251 2005) was used for detection and enumeration of *E. coli*. Ten milliliter of the initial suspension was inoculated to 10 ml double concentration tubes of lauryl tryptose broth, as selective enrichment medium (Liofilchem 610085, Italy), and 1 and 0.1 ml of the suspension were inoculated to 9 ml of normal concentration of this medium and were incubated at 37 °C for 24 h.

In the next step, the tubes containing EC broth selective medium (Merck 1107650500, Darmstadt, Germany) were inoculated in a suspension containing culture medium. After observing gas or opacity, they were incubated in a water bath at 44 °C for 24 h. A loop of medium from each tube was then inoculated in the tubes containing peptone water (Titan Biotech M028, India) and was incubated in a water bath at 44 °C for 48 ± 2 h. Finally, 0.5 ml of indole reagent was added to the incubated pepton water tubes, well stirred and was checked after one minute. Observation of the red wine color in the alcoholic phase (indole production) was considered as growth of *E. coli*. For each dilution, the number of positive tubes with double and normal concentration was reported, using MPN table.

For enumeration and identification of the intestinal enterococci, 1 ml of the initial suspension was added into the sterile plates and was then mixed with 15–20 ml of melted KF-streptococcus agar (Merck 1.10707.0500, Darmstadt, Germany) containing 1% 2,3,5–triphenyl-2*H*-tetrazolium chloride (TTC) solution (Merck 1083800010, Darmstadt, Germany). After solidification it was incubated at 35–37 °C for 24–48 h and red, pink and purple colonies on the plate surface of the medium were counted as the fecal enterococci. To confirm the intestinal enterococci, the suspected colonies were streaky cultured on bile-escualin agar plates and were incubated at 44 °C for 2 h. When the color of the medium was changed to tan or black, the colonies were considered as intestinal enterococci and were reported CFU per gram (ISIRI 2198 2008).

For total count of microorganisms at 30 °C, 1 ml of the initial suspension (0.1 dilution) and subsequent dilutions (0.01 and 0.001) the microorganisms were transferred into each of the two sterile plates. Fifteen ml of plate count agar medium (Liofilchem 610040, Italy) was added to each plate and was incubated at 30 °C for 72 ± 3 h. After incubation, the colonies were counted and the mean numbers of the two successive dilutions were reported in CFU/gr (ISIRI 5272 2007; ISO 4833-1 2013).

To isolate the *Salmonellas* spp. (ISIRI 1810 2002; ISO 6579 2002), the primary suspension (25: 225 g/ml) was

incubated at 37 °C for 16-20 h. Deci ml (0.1) of the preenrichment broth was inoculated to 10 ml of the Rappaport-Vassiliadis Soy (RVS) broth (Liofilchem 610175, Italy) and 1 ml of it was inoculated to 10 ml of Muller-Kauffmann Tetrationate Novobiocine enrichment broth (Merck 1058780500, Darmstadt, Germany) and was incubated at 41.5 °C for 24-48 h. Each of the two enriched environment were cultured streaky on Brilliant-green phenol-red lactose sucrose agar (BPLS) medium (Merck 1072320500, Darmstadt, Germany) and Salmonella-Shigella agar (SS agar) (Merck 1076670500, Darmstadt, Germany) and were incubated at 37 °C for 48 h. The dubitable colonies in the previous step were selected and were cultured on nutrient agar (Liofilchem 610036, Italy). The plates were incubated at 37 °C for 18-24 h. Salmonella was confirmed using biochemical tests and TSI (Liofilchem 610055, Italy), LIA (Liofilchem 610027, Italy), urea agar (Liofilchem 610107, Italy), SIM (Liofilchem 610181, Italy), Simmons citrate (ATD M069, UK), Trypton water (Himedia RM014, India), lysine decarboxylase (Liofilchem 610303, Italy) and MRVP media (Liofilchem 610032, Italy). The microorganisms were incubated at 37 °C for 24 h. Then latex agglutination applied to id entification of Salmonella.

Each test was repeated three times.

Histopathological study

Histopathological examination was carried out by an expert pathologist. Five infected and none infected MLNs with *L. serrata* were fixed in 10% neutral buffered formalin (Merck 1039992500, Darmstadt, Germany), dehydrated in graded ethanol, and embedded in paraffin. Sections of 5 μ m in thickness were stained with Hematoxylin and Eosin (H & E) (Oryan et al. 2011) and observed by a light microscope for the presence of parasite nymphs and bacterial agents (cocci, rods, etc.).

Statistical analysis

Statistical analysis was performed, using SPSS version 18, by an independent *t* test and P < 0.05 (95%) was considered as significant difference.

Results

Bacterial contamination

The average total bacterial count and *E. coli* count in both parasite positive/negative samples are shown in Figs. 2 and 3 Comparison between the two groups demonstrated that the parasite positive lymph nodes showed significantly

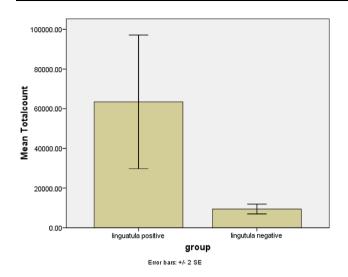


Fig. 2 Mean CFU/g of total count in *Linguatula* positive and nagative MLNs samples

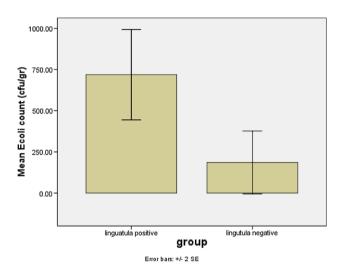


Fig. 3 Mean MPN/g of *E. coli* in *Linguatula* positive and nagative MLNs samples

higher bacterial infection than the parasite negative ones (P < 0.05). Colonies of *Salmonella* spp. were observed among four parasite positive (36.36%) and one parasite negative (9.09%) samples. There was no statistically significant difference in the presence of *Salmonella* and fecal enterococci between two groups (P > 0.05). However, except one parasite negative sample, the intestinal enterococci were not confirmed in other parasite positive/negative samples.

Histopathological findings

The histopathological sections in the parasite positive lymph nodes showed various stages of acute and/or chronic inflammation with infiltration of neutrophils, eosinophils, macrophages, lymphocytes, plasma cells and

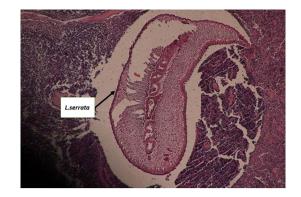


Fig. 4 Longitudinal sections of *L. serrata* in a mesenteric lymph node (accumulation of various bacterial forms including cocci and bacilli were seen in the tissue surrounding the parasite nymph)

giant cells in vicinity of the *L. serrata* larva. Some sections showed proliferation of fibroblasts and formation of fibrous connective tissue in the infected area while some others showed mineralization of the parasitic larva and necrosis of the infected lymph nodes. In some instances the peripheral spines of the larva of *L. serrata* resulted in severe damage of the infected lymph nodes. In addition to the above pathological changes, accumulation of various bacterial forms including cocci and bacilli were clearly seen in the tissue surrounding the parasite nymph. In most cases of parasite negative lymph nodes were either free of bacterial contamination or showed lower bacterial contamination compared to those of parasite positive ones (Fig. 4).

Discussion

The aim of this study was to investigate the role of *L. serrata* nymph in bacterial contamination of the animal products. It was demonstrated that there was a significant difference in the total bacterial counts between parasite positive/negative samples. So that the average total count in parasite positive samples was 6.7 times higher than the parasite negative samples.

Based upon the histopathological findings and the higher bacterial infection in the parsite positive tissues, presence of the *L. serrata* nymph seems to result in an increase of bacterial contamination of the infected organs. This can indicate that the *L. serrata* nymphs can transfer some of the organisms of the intestine to the target tissues and contaminate these organs with the intestinal bacteria. Miclaus et al. (2008) have reported evidence of aggregation of bacilli and cocci forms of bacteria in the MLNs of sheep based on histopathological study and concluded that this had occurred due to migration of *L. serrata* from the intestine to other organs. Therefore, on the basis of this study it can be concluded that the inoculative effect of *L*. *serrata* to transfer bacteria is very likely.

Svanevik et al. (2013) confirmed the role of *anisakid* larva on bacterial contamination of the flesh of post-harvest blue whiting. They concluded that this effect was due to transfer of the larva from intestine to flesh with similar mechanisms. They similarly showed that the total bacterial count in the infected group was seven times more than the uninfected group.

In recognition of the possible inoculative effects, it is also important to note that spoilage agents may also be transmitted by this parasite. As Miclaus et al. (2008) evaluated the inoculative action of L. serrata by histopathological study and showed presence of yeast cells around L. serrate nymphs. Mir et al. (2009) reported concurrent occurrence of visceral linguatulosis and paratuberculosis (Mycobacterium avium sub species paratuberculosis) in alpine cross goats, which suggests the possibility of inoculative effect of L. serrate to transfer bacterial agents. As well in a study in 2009 that was conducted with histopathological and bacteriological method in cattle livers, also stated that *Fasciola* plays an important role in the microbial invasion of the infected animals either by transportation or by depressing the vital resistance of the host (Sohair and Eman 2009).

There is possible of spoilage bacteria transmission by *L. serrata* to mesenteric lymph nodes, liver, lungs and possibly other internal organs. Therefore, presence of parasitic can cause more contamination and faster spoilage of these products. However, further investigations are required to review and approve this subject. Svanevik et al. (2014) showed that the fish mince contaminated with bacteria which originate from *Anisakis* larvae, spoiled less rapidly than samples without any parasite-related bacteria presence.

The present study showed a significant difference between the mean count of *E. coli* between parasite positive and negative samples. Comparison of the mean count of *E. coli* in the two groups revealed that pollution in parasite positive samples was 3.3 times higher than those of parasite negative samples. *Escherichia coli* is one of the most important pathogens in most cases of foodborne infections. Enterohemorrhagic *E. coli* (EHEC), particularly the O157: H7 serotype is known as one of the causative agents of hemorrhagic colitis and uremic hemolytic syndrome (Adams and Moss 2008), thus with confirming the inoculative effect of *L. serrata*, the risk of consumption of the parasite positive lymph nodes is doubled.

Enterococci have been implicated in cases of food poisoning by production of biogenic amines, based on their isolation in high numbers from the suspect foods, but this statement still has not found direct support (Giraffa 2002). In present study intestinal enterococci were isolated from one parasite negative lymph node. However, in all cases, suspected colonies in KF-streptococcus agar medium were counted.

All food animals are susceptible to infection with Salmonella, belonging to the family Enterobacteriaceae. Infected animals that do not develop salmonellosis, and those that recover from the disease, become carriers of Salmonella and serve as sources of infection to humans and other animals (Ekperigin and Nagaraja 1998). Presence of Salmonella was confirmed in few lymph nodes, in the present study, and significant differences were not statistically found between the two groups. However, higher infection rate of Salmonella infection has been reported in the MLNs of animals in a study which showed the presence of Salmonella contamination in the lymph nodes of slaughtered animal from 4 to 88.2% (Haneklaus et al. 2012). Therefore, it is possible that salmonella spp. are transmitted by the Linguatula nymphs from intestine to mesenteric lymph nodes, liver and possibly other infected internal organs.

In terms of food safety, confirmation of the inoculative effect of *L. serrata* nymphs in transmission of bacteria is important. *L. serrata* nymphs also migrate to other organs such as the liver, lungs, heart, kidneys, spleen and other parts of the body, in addition to the mesenteric lymph nodes. Therefore, linguatulosis results in higher bacterial infection of these organs. Considering that the consumption of raw offal, especially liver and MLNs are common in some regions, and this parasite is considered a potential risk for these people, there is possibility that such people get bacterial infection by consuming the raw or uncooked edible organs infected with *L. serrata*.

Linguatula infection may also transfer anaerobic bacteria and also Mycobacterium tuberculosis, M. bovis, M. paratuberculosis and so on. Such bacterial contamination also should be examined in relation to Linguatula infection in further investigations. It is suggested that the possible role of this parasite in transmission of other important pathogens, particularly Campylobacter and spoilage bacteria in other target tissues such as the liver and lungs is examined in future studies.

Conclusions

With regard to the consumption of raw infected lymph nodes in some countries, there is the potential of illness in people. Consequently, in addition to the risk of parasite in the lymph nodes with *L. serrata* (Halzoun syndrome), the risk of bacterial diseases should also be considered in such instances.

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Complaince with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

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