JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Brief Original Article

High seroprevalence of pathogenic *Yersinia* spp. in sheep and goats across nine government farms in the Pakistani Punjab

Qudrat Ullah[1,2], Tariq Jamil[2], Muhammad H Hussain[3], Huma Jamil[1], Muhammad Saqib[4], Usman Tahir[5], Heinrich Neubauer[2], Lisa D Sprague[2]

[1] Department of Theriogenology, University of Agriculture, Faisalabad, Pakistan
[2] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Naumburger, Jena, Germany
[3] Animal Health Research Center, Ministry of Agriculture and Fisheries, Muskat, Oman
[4] Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan
[5] Livestock and Dairy Development Department (L&DD), Anarkali, Lahore, Punjab, Pakistan

Abstract

Introduction: Seroprevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* infections in animals and humans is not established in Pakistan. There are only a few reports on the prevalence of pathogenic *Yersinia* spp. and infections in small ruminants, however, the role of sheep and goats in the transmission of pathogenic *Yersinia* remains unclear.

Methodology: A primary survey investigated the presence of anti-*Yersinia* antibodies among a small population of ruminants detected by recombinant antigen targets in nine government farms dispersed throughout the Punjab province of Pakistan.

Results: Antibodies specific for *Y. enterocolitica* were detected in 7/9 sheep flocks and in 4/4 goat flocks. Antibodies specific for *Y. pseudotuberculosis* were detected in 4/9 sheep flocks. Two sheep flocks revealed the presence of both *Y. enterocolitica* and *Y. pseudotuberculosis* specific antibodies.

Conclusion: Due to the high number of the population involved in raising small ruminants the risk to veterinary and public health must be rapidly determined.

Key words: Yersinia, serology; small ruminants; Punjab; Pakistan.

J Infect Dev Ctries 2019; 13(9):843-846. doi:10.3855/jidc.11289

(Received 30 January 2019 – Accepted 23 May 2019)

Introduction

Yersinia (*Y.*) *enterocolitica* and *Y. pseudotuberculosis* are the causative agents of yersiniosis, which is mostly a self-limiting gastrointestinal disease in humans and animals. Yersiniosis is one of the most common but largely underdiagnosed bacterial zoonosis observed in all continents, exceeded only by salmonellosis and campylobacteriosis [1-5]. Pigs are considered to be the main reservoir for human pathogenic strains [6]. Pathogenic Yersinia (*Y. enterocolitica Y. pseudotuberculosis* and *Y. pestis*) species all possess a Yop virulon, which is encoded on the 70 kb Yersinia virulence plasmid (pYV). This Yop virulon is made up of Yop effector proteins and other proteins necessary for introducing the virulon into the host cell. Once in the host cell, these virulons interfere with the cytoskeleton, disrupting phagocytosis, and preventing the production of cytokines thereby enabling the survival of the invading pathogen [7]. Further chromosomally encoded virulence factors, such as *ail* and *inv* facilitate invasion, whereas different Yersinia stable toxin (*yst*) genes are responsible for the production of heat-stable enterotoxins, found in both enteropathogenic *Y. enterocolitica* biotypes (1B, 2-5) and the allegedly apathogenic bt 1A [8].

There are only a few reports on the prevalence of pathogenic *Yersinia* spp. and infections in small ruminants, however, the role of sheep and goats in the transmission of pathogenic *Yersinia* remains unclear. [9-11]. Sheep and goats are a major source of income in developing countries and transition economies such as Pakistan. The rapid growth of Pakistan's population has led to a rise in the demand for fruit and vegetables but also for dairy products and meat. According to the Ministry of National Food Security and Research, there are approximately 30 million sheep and 72 million goats in Pakistan and nearly eight million families are

involved in livestock rearing and production activities (http://www.finance.gov.pk/survey_1617.html, Accessed: 22 September 2019). In 2016/2017, the estimated milk production of sheep and goat origin was approx. 40,000 and 900,000 tons, respectively, and for mutton meat production approx. 700,000 tons (http://www.finance.gov.pk/survey/chapters_17/02-Agriculture.pdf; accessed 19, October 2018). As there are no data available on the prevalence of pathogenic *Yersinia* spp among the population of small ruminants in Pakistan, a primary survey was conducted among nine government farms located in seven districts; Kushab, Bhakkar, Layyah, Rajanpur, Bahawalpur, Khanewal, and Okara of the Pakistani Punjab.

Methodology

Sample collection

A total of 1000 serum samples from sheep and goats were collected from nine government farms in the Pakistani Punjab stratified by sampling and proportionate allocation [12]. For blood collection, guidelines of the International Animal Care and Use Committee (IACUC) were followed. Approval was given by the Secretary of the Livestock & Dairy Development Department, Punjab, Pakistan (letter No. SO(I&C)/L7DD/2-6/2016). Ethical approval was received from the Ethical Research Board at the University of Agriculture Faisalabad, Pakistan (letter No. ORIC/3253, 16.11. 2013).

Blood was collected from the jugular vein using Improvacuter Blood Collection Sets (Improve Medical, Gangzhou, China). Each tube was labelled immediately after collection and placed vertically in a cool box with cool packs. Samples were then transferred to the laboratory in Faisalabad and subjected to centrifugation (4500 rpm/5 minutes/4°C). The sera were transferred to collection tubes (CryoS^{TM}, Greiner Bio-one, Frickenhausen, Germany) and subsequently shipped to the Friedrich-Loeffler-Institut in Jena, Germany. Samples were stored at -20°C until further use.

Western Blot

A total of 388 samples were tested for the presence of anti-*Yersinia* antibodies (IgG) using the recomLine Yersinia IgG 2.0 Kit (Mikrogen Diagnostik, Neuried, Germany) according to the manufacturer's instructions (Table 1). This test uses recombinantly produced antigen targets, i.e. *Yersinia* outer proteins YOP-M, YOP-D, YOP-E; V-AG, PsaA (pH 6 antigen) and MyfA (Mucoid Yersinia factor A). Briefly, 20 µL of serum was added to test strips placed into incubation trays with 2 mL of wash buffer. The strips were incubated under gentle shaking for one hour at room temperature (RT) and subsequently washed three times with wash buffer for 5 minutes under gentle shaking at RT. Next, the strips were incubated in wash buffer and the corresponding antibody (rabbit-anti-sheep-peroxidase (POD) or rabbit-anti-goat-POD; (Dianova, Hamburg, Germany) for 45 minutes under gentle shaking at RT. After three rinsing steps with wash buffer the strips were incubated in substrate solution. The reaction was stopped after development of bands or after 8 minutes. The strips were washed with distilled water and placed between absorbent tissues and stored in the dark until evaluation. Analysis of the test was carried out after checking for the clearly stained reaction control band, the antibody conjugate control band (here: IgG) and the presence of the cut-off control band. Next, band intensity for the respective antigens was assessed in relation to the cut-off band. Only bands with intensities equivalent or stronger than the cut-off band were considered positive. Tests were considered

Table 1. Summary of farms tested in different districts of the Punjab Province, Pakistan.

Farm	Species	Flock size	Samples taken	Samples tested	mean age in months	Females	Males	Yop D	Yop E	Yop M	V-AG	MyfA	PsaA
1	sheep	383	40	31	45.6 (10-108)	28	3	30	28	30	18	9	0
	goat	281	203	35	51.7 (10-122)	30	5	35	34	35	20	26	0
2	sheep	90	9	9	55.2 (14-88)	9	0	9	9	9	6	7	0
	goat	60	43	35	40.1 (10-108)	25	10	35	35	35	15	29	0
3	sheep	710	73	31	45.6 (11-108)	31	0	30	20	29	12	8	0
	goat	290	210	35	52.6 (8-108)	11	24	34	24	34	13	11	0
4	sheep	848	88	31	39.6 (13-108)	31	0	31	31	31	24	0	12
	goat	61	44	35	51.7 (2-86)	30	5	35	34	34	32	4	0
5	sheep	430	45	31	43.2 (23-83)	30	1	31	22	30	14	18	0
6	sheep	356	37	31	57.6 (9-132)	30	1	29	29	31	27	0	19
7	sheep	1400	145	31	52.8 (12-89)	31	0	31	31	31	30	2	14
8	sheep	214	22	22	39.6 (11-88)	19	3	22	16	22	13	3	6
9	sheep	400	41	31	52.8 (6-95)	30	1	31	14	30	10	14	0

f: female; m: male.

positive if the anti-Yop D band and one further antigen band (Yop M, V-AG, Yop M) reacted. Differentiation for *Y. pseudotuberculosis* and *Y. enterocolitica* in positive tests was done according to the following criteria: For *Y. pseudotuberculosis*: PsaA band ≥ cut-off and MyfA band < cut-off. For *Y. enterocolitica*: MyfA band ≥ cut-off and PsaA band < cut-off. If none of the criteria applied, a differentiation between the species was not possible.

Results

In total, 388 samples were tested (248 sera from sheep and a total of 140 sera from goats) for the presence of anti-*Yersinia* antibodies (Table 1). The flocks had a history of reproductive disorders such as stillbirth, premature delivery, weak lambs/kids and abortion. The age of the sampled animals ranged six months to 11 years and two months to 9 years among sheep and goats, respectively. The youngest positive animal (goat) was 2 months old; the oldest animal (sheep) was 11 years old. Antibodies specific for *Y. enterocolitica* were detected in 7/9 sheep flocks and in 4/4 goat flocks. Antibodies specific for *Y. pseudotuberculosis* were detected in 4/9 sheep flocks. Two sheep flocks revealed the presence of both *Y. enterocolitica* and *Y. pseudotuberculosis* specific antibodies.

Discussion

Seroprevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* infections in animals and humans is not established in Pakistan. Due to the worldwide occurrence of enteropathogenic *Yersinia* spp. we assumed that yersiniosis should also be present in Pakistan. A large population of small ruminants in Pakistan and a significant number of people involved in livestock rearing and production activities may pose a significant health risk regarding yersiniosis. Moreover, *Y. enterocolitica* O:9 serovars might also hamper the serological detection of *Brucella abortus*, the causative agent of the highly contagious zoonosis Brucellosis, due to possible cross-reactions [13,14]. A primary survey was therefore conducted at government farms testing the presence of anti-*Yersinia* antibodies in goats and sheep using the recomLine Yersinia IgG 2.0 kit. This assay targets antibodies against the *Yersinia* outer proteins Yop-M, Yop-D, Yop-E as well as V-AG, PsaA and MyfA. Using target proteins specifically associated with *Yersinia* pathogenicity for serology reduces the number of false positivity in comparison to the lipopolysaccharide (LPS) derived antigens [15]. Only a few studies have been conducted analysing the presence of pathogenic *Yersinia* spp. in small ruminants. A cohort study from New Zealand analysing goat stool samples revealed that asymptomatic carriage of *Yersinia* infections was common. Over a one-year study period, 21% of the cultured faecal samples were positive for *Y. enterocolitica* biotype (bt) 5, O:2,3, 6% positive for *Y. enterocolitica* bt 1A, 3 % positive for *Y. enterocolitica* bt 3, O:5,27, 14% positive for *Y. pseudotuberculosis* and 36% positive for *Y. frederiksenii* [9]. A study from Iran on *Yersinia* spp. in raw milk from goat and sheep revealed a prevalence of 2.4% and 3% for *Y. enterocolitica* bt 1A, O:9, respectively [16]. Up to 34% mortality has been reported among sheep due to yersiniosis caused by *Y. enterocolitica* bt 3 in China [17]. An Australian study on prevalence and faecal shedding of *Yersinia* spp. in sheep determined point prevalence ranging from 4% to 91% across farms and sampling occasions, with up to 60.7% of lambs shedding *Yersinia* spp. on at least one sampling occasion. This study detected four species, i.e. *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. intermedia* and *Y. frederiksenii* detected by qPCR [11]. In a prospective study conducted between 1996 and 1999 in the Indian Punjab on *Y. enterocolitica* enteritis, *Y. enterocolitica* was isolated in 2% of human stool samples, with bt 4, O:3 being the most prevalent [18].

Conclusion

The present primary study has shown for the first time that small ruminants in the Pakistani Punjab are exposed to pathogenic *Yersinia* spp. Due to a significant number of the people involved in raising small ruminants, further studies are urgently needed to determine the threat of yersiniosis to the veterinary and public health and to characterise the circulating strains with regard to their antibiotic susceptibility.

Acknowledgements

The authors would like to thank the staff at the government farms for their help. This work was financed by the Higher Education Commission, Islamabad, Pakistan under the International Research Support Initiative Program (IRSIP), Grant # 12201-0539230-9.

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Corresponding author

Dr. Lisa D. Sprague
Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96a, 07743 Jena, Germany
Phone: 0049 3641 8042462
Fax: 0049 3641 8042228
Email: lisa.sprague@fli.de

Conflict of interests: No conflict of interests is declared.