IJBPAS, January, 2016, 5(1): 271-284

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

HEAT SHOCK PROTEIN 70 (HSP70): A POTENTIAL BIOMARKER FOR CAMPYLOBACTERIOSIS

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ABSTRACT

Campylobacter species are the most common bacterial cause of human gastroenteritis worldwide and prevalent in developing countries. Most infections occur during infant stage but also in adult by consumption and handling of poultry. Conventional methods like cultures from stool samples are used to detect campylobacters among ostrich's farms and the zoo. *Campylobacter jejuni* (18%) and *Campylobacter coli* (6.67%) could be detected among the examined samples. The aim of the present study was to estimate HSP70 in serum of the rats infected with campylobacters. Fifty five adult male Wistar rats at 10 weeks of age were randomly assigned to 11 groups (5/group): 5 groups injected with *C. jejuni* and 5 groups injected with *C. coli* strains and the last group was left separately without any injection as negative control. Histopathological findings were identified among the experimentally infected rats. Blood samples of the experimental rats were taken at day 7 and day 14 days

after infection and serum HSP70 levels were measured using enzyme-linked immunosorbent assay (ELISA). Significantly high levels of HSP70 were detected in serum from rats infected by *C. jejuni* and *C. coli* (0.68 and 0.84 ng / ml respectively) at day 7 post infection as compared to the control group (0.2 ng / ml).

In conclusion, the level of HSP70 was remarkably increased in the infected rats compared with those in control which can be a potential biomarker for campylobacteriosis.

Keywords: Campylobacteriosis, ELISA, Histopathology, HSP70, Biomarker

INTRODUCTION

Wild and domestic birds act as major reservoirs of *Campylobacter* species and play a role in epidemiology of the disease (Wedderkopp *et al*., 2003). Recent studies have suggested a potential role for birds in campylobacter spp. transmission, which is the leading cause of gastroenteritis in humans (Weis *et al*., 2014) .The thermophilic campylobacters, have been isolated from many species of birds including ostriches. The consumption of campylobacter contaminated food and water causes gastrointestinal illness in human (El-Jakee *et al*., 2008). The most common cause of human infection is *Campylobacter jejuni*, followed by *Campylobacter coli*, but *Campylobacter lari*, *Campylobacter fetus* and *Campylobacter upsaliensis* have also been reported to cause human infections (Patrick *et al*., 2013). Commonly reported symptoms of campylobacteriosis include diarrhea, abdominal pain, fever, malaise, nausea and headaches.

Campylobacteriosis is approximately affecting 1.3 million people in the United States alone and in rare cases; infection can also lead to serious disorders and sysdromes (Nachamkin. 2003, Weis *et al*., 2014). Guillain-Barré syndrome (GBS) is a disease of the nervous system which is caused by *C. jejuni* infection and can result in acute neuromuscular paralysis. Irritable bowel syndrome is anther sequel to campylobacteriosis that causes significant social and economic burden (Mughini *et al*., 2012). Similarly, approximately 1% of campylobacteriosis also affected with Reiter's syndrome; a reactive arthritis that can affect multiple joints causing pain and incapacitation (Ternhag *et al*., 2005).

Campylobacter has developed resistance to several antimicrobial agents and becoming a significant public health hazard (Di Giannatale *et al*., 2014).

Heat shock proteins (HSPs) are molecular chaperones that are widely distributed in nature, and are highly conserved proteins among prokaryotes and eukaryotes. HSPs

were first discovered in 1962 as a set of highly conserved proteins whose expression was induced by different kinds of stress (Ritossa, 1962). The principal HSPs that have chaperone activity belong to five conserved classes according to their molecular weight: HSP33, HSP60, HSP70, HSP90, HSP100, and the small heat-shock proteins (Schlesinger, 1990). Exposure of cells to microbial pathogens can induce HSPs, which prevent protein aggregation and facilitate folding. HSP70 has been shown to have an important role in numerous diseases (Evans *et al.*, 2010). Schwann and Goebel (1994) recorded that the bacterial infection is known to be a potent inducer of HSP70 expression in variety of cell types that help to protect the bacterial cell against stress factors.

The current work is established to study ecological monitoring of *Campylobacter* species in ostrich farms along with the zoo and to detect heat shock protein 70 (HSP70) in serum of rats infected by campylobacters isolated from ostriches using ELISA.

MATERIALS AND METHODS

Samples for culture

Total 150 sample of Fecal (n= 102), water (n= 36) and food (n= 12) were collected from ostrich private farms (n= 77), governmental farms (n= 27) and the city zoo (n= 46) for isolation of *Campylobacter* species. All samples were collected in sterile tubes and cultured within two hours.

Isolation and Identification of *Campylobacter* species

According to Boonmar *et al.* (2005) protocol, and ISO 10272 -2006 standard, 1 g or ml of each sample was added to 9 ml of Preston *Campylobacter* selective enrichment broth (Oxoid). This enrichment broth was supplemented with *Campylobacter* selective supplement SR 117 (Oxoid), *Campylobacter* growth supplement SR 84 (Oxoid) and 5% defibrenated horse blood. The inoculated broth was incubated under microaerophilic conditions at 42ºC for 24 hours. Then the enrichment culture was streaked onto *Campylobacter* blood-free selective agar base (Oxoid) supplemented with *Campylobacter* selective supplement SR 155 (Oxoid) and further incubated under microaerophilic conditions at 42ºC for 3-5 days. Suspected *Campylobacter* colony was confirmed using standard biochemical procedure including catalase, oxidase and hippurate hydrolysis tests (Nachamkin, 2003).

Experimental infection of rats with *Campylobacter jejuni* and *Campylobacter coli* isolates

A total number of 55 adult male Wistar rats weighing about 235-270 g were injected with campylobacters at age of 10 weeks.

The rats were divided to total 11 groups with 5 animals per group: group was left separately without any injection (control negative), group was injected with the standard strain of *Campylobacter jejuni* ATCC 33291 (control positive J), group was injected with the standard strain of *Campylobacter coli* ATCC 33559 (control positive C), 4 groups were injected with 4 *Campylobacter jejuni* isolates, and 4 groups were injected with 4 *Campylobacter coli* isolates. Each rat in the infected groups was injected intraperitoneum with 1 ml of the prepared *Campylobacter* suspension (3×10^9 CFU/ml). The injected rats were kept under the observation for 2 successive weeks. Animals care as well as experimental protocols was incompliance with guidelines of ethical standards released by Cairo University policy on animal care and use.

Blood samples

Blood samples were collected at day 7 and day 14 post infection from the medial canthus of the rat's eye in a sterile vacutainer tubes. The blood samples were centrifuged at 4500 rpm for 20 minutes at 4°C to remove any traces, clots or debris and obtain clear serum. The sera were preserved in cryovials and stored at -20°C until further used.

Histopathological examination

The liver and intestine samples of the rats infected with *Campylobacter jejuni* and *Campylobacter coli* were collected from specific groups at the day 14 post injection and subjected to re-isolation of campylobacters and histopathological examination according to (Downie, 1990).

Detection of HSP70 among the infected rats using ELISA

Enzyme-linked Immunosorbent Assay Kit (E9087Ra 96 tests, Uscn Life Science Inc.) is a sandwich enzyme immunoassay for the in vitro quantitative measurement of HSP70 in rat serum. ELISA was done using kit according to the manufacturer's instructions and House *et al*. (1993). The color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm using ELISA reader (TECAN). The standard curve concentrations used for the ELISA's were 10 ng /ml, 5 ng /ml, 2.5 ng/ml,1.25 ng /ml, 0.625 ng /ml, 0.312 ng /ml, 0.156 ng/ml. The concentration of HSP70 in the samples was determined by comparing the optical density O.D. of the samples to the standard curve.

RESULTS

The occurrence of *Campylobacter* species among the apparently healthy ostrich and their environment

A total of one hundred and fifty samples (102 feces, 36 water and 12 food samples)

were collected from private and governmental ostrich's farms and the zoo for campylobacters isolation. 37 out of 150 samples were revealed positive for *Campylobacter* species (24.67%) and used for *Campylobacter* isolation. It is clear that the highest rate of *Campylobacter* isolates were from water samples collected from the zoo (57.14%) followed by food samples collected from private ostrich's farms and the zoo (33.33% each), water samples collected from private ostrich's farms (31.25%), then fecal samples collected from the zoo, private and governmental ostrich's farms (30.77, 18.97 and 11.11% respectively). While *Campylobacter* species could not be detected from food and water samples collected from governmental ostrich's farms Figure 1. The highest percentage of *Campylobacter jejuni* was identified from the zoo samples (36.96%) followed by private farms (10.39%) and governmental farms (7.41%). The highest percentage of *Campylobacter coli* was identified from private farms (11.69%) followed by the zoo samples (2.17%) while it couldn't be isolated from the governmental farms (Figure 2). As shown in Figure 3, *Campylobacter jejuni* was identified from the examined food and water samples (25% each) and from 14.71% of feces collected from apparently healthy ostriches. As compared to *Campylobacter jejuni*, *less* isolates of *Campylobacter coli* was identified from water samples (11.11%) and from feces collected from apparently healthy ostriches (5.88%), while it couldn't be isolated from food samples. In Figure 4, it was cleared that the highest incidence rate of *Campylobacter* species were obtained during warmer seasons (spring and summer) other than cold season (winter).

The pathological and histopathological examinations

The rats injected with campylobacters were kept under the observation for 2 successive weeks. During the first week after infection the rate showed some signs of loss of appetite, restlessness and softening of feces.

The symptoms among the rats injected with *Campylobacter jejuni* showed high level of morbidity and clinical signs other than that injected with *Campylobacter coli*. In the second week these signs were decreased and there were no deaths in between the rats all over the 2 weeks. The liver and intestine of the infected rats showed edema and necrotic foci (Figure 5). After 14 days of infection, the liver and intestine were examined histopathologically. The fixed liver and intestine samples had histopathological lesions as shown in Figures 6 and 7.

Results of rat Heat Shock Protein 70 (HSP70) using ELISA

The HSP70 readings in rat sera were estimated at the first and second week post infection with *C. jejuni* and *C. coli* as shown in Table 1.

It is clear that there is significant increase in the HSP70 among the infected rats than the uninfected group.

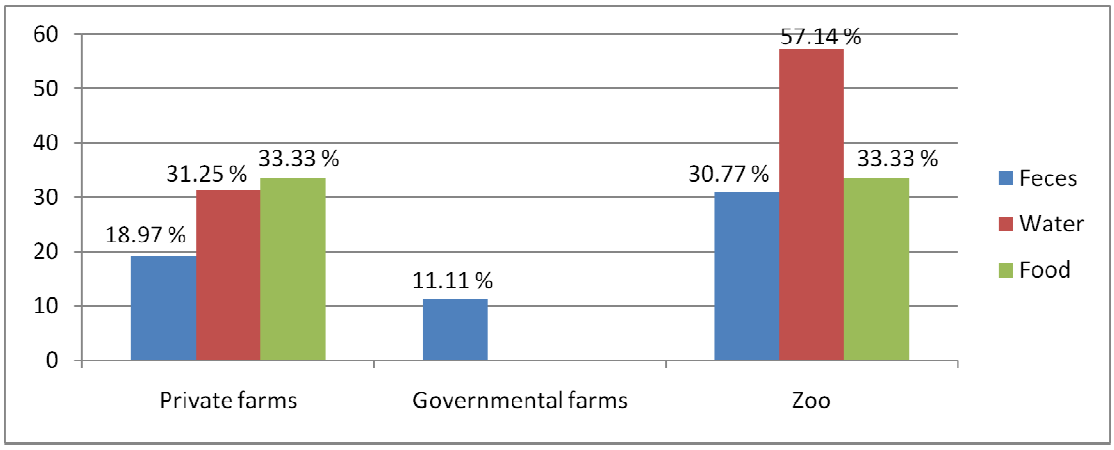

Figure 1: The occurrence rate of *Campylobacter* species among ostrich's farms and the zoo

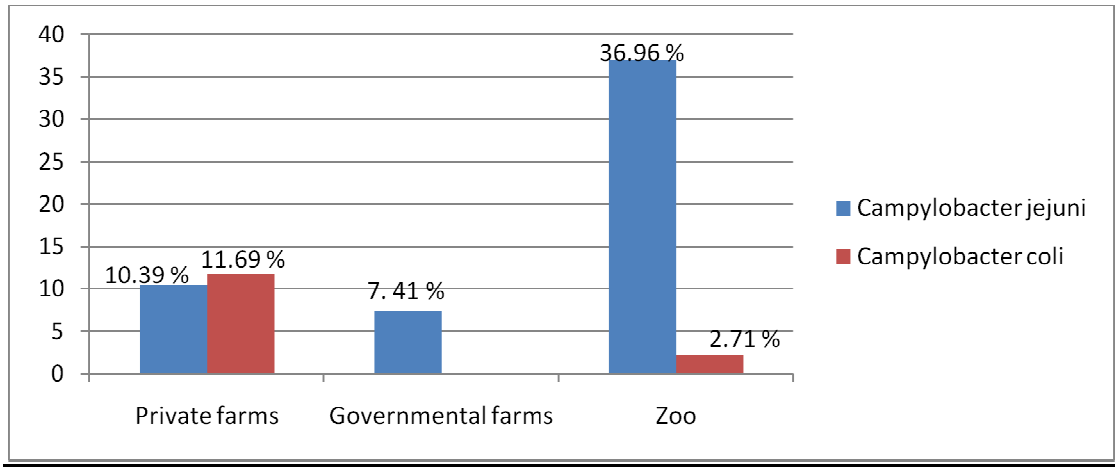

Figure 2: The occurrence rate of *C. jejuni* and *C. coli* in ostrich's farms and the zoo

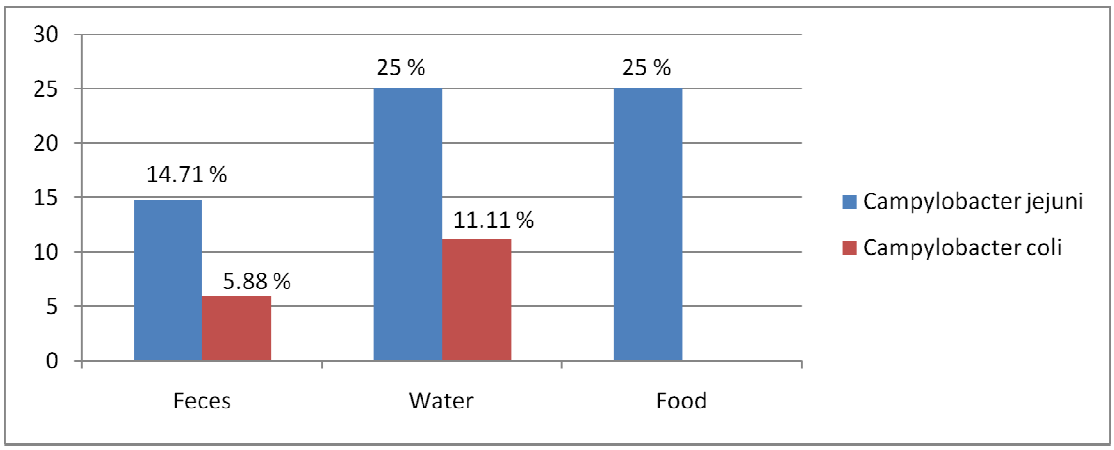

Figure 3: The occurrence rate of *C. jejuni* and *C. coli* among the examined samples

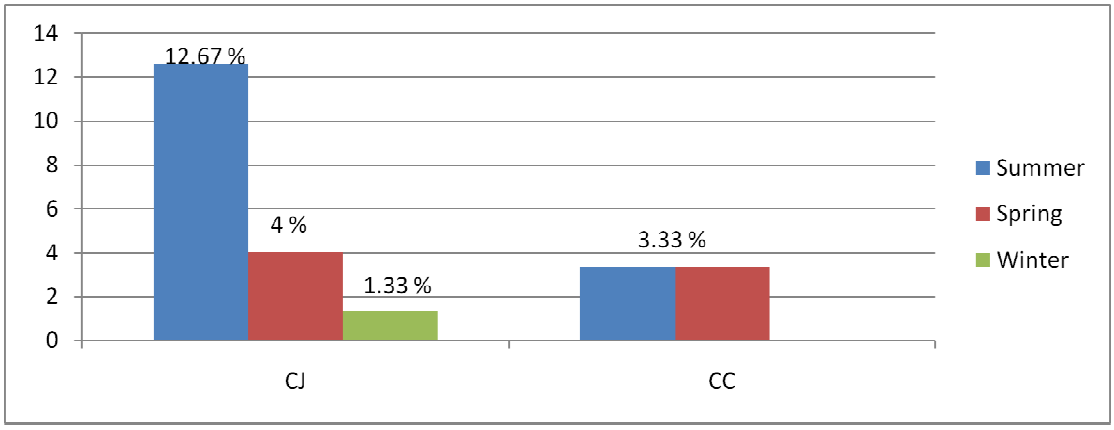

Figure 4: The incidence of *Campylobacter jejuni* (CJ) and *Campylobacter coli* (CC) among the examined farms regarding to seasonal variations

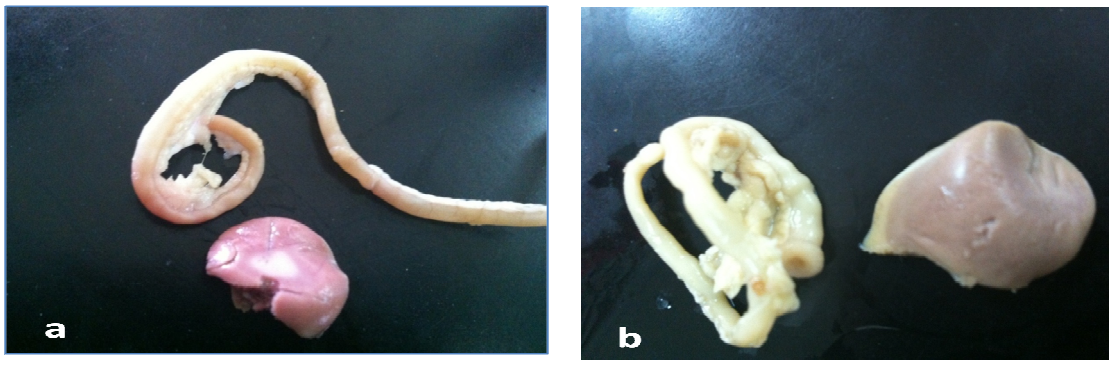

Figure 5: The rat liver and intestine after injection with *Campylobacter* species.
a): The rat liver and intestine after injection with *Campylobacter jejuni.* The rat liver was swollen with rounded edges and multifocal whitish grey focal necrotic lesions. Edema, paleness and thickened folds were detected in the intestine.
b): The rat liver and intestine after injection with *Campylobacter coli.* There were white necrotic foci at liver edges. Edema and paleness were detected in the rat intestine

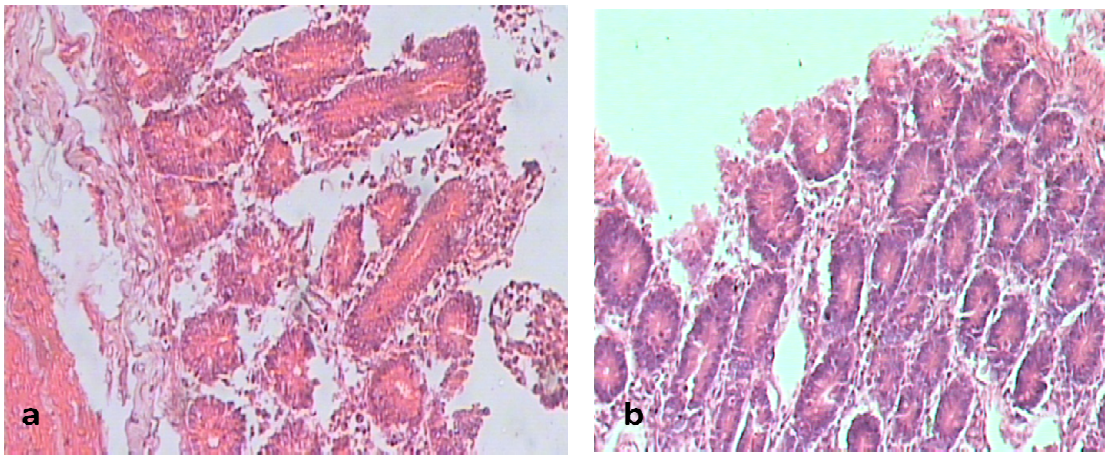

Figure 6: The Intestine of rat injected with *Campylobacter* species.
a): The Intestine of rat injected with *Campylobacter jejuni* showing destruction and necrosis of some intestinal glands with desquamation of epithelial cells covering superficial surfaces of the villi (H& E 100). b): Intestine of rat injected with *Campylobacter coli* showing destruction of some intestinal gland and desquamation of the lining epithelial surface with infiltration of mononuclear inflammatory cells and neutrophils (H &E 100).

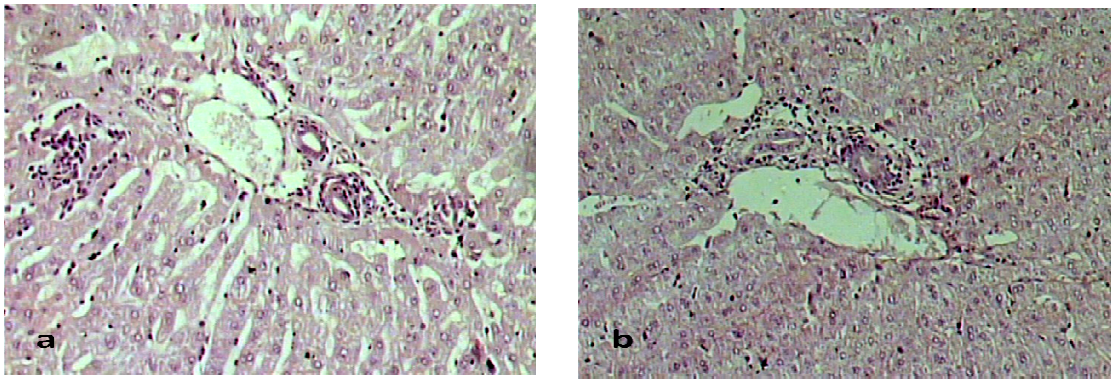

Figure 7: Liver of rat injected with *Campylobacter* species.
a): Liver of the rat injected with *Campylobacter jejuni* showing foci of lymphocytic infiltrations and necrosis of hepatocytes with inflammatory cell aggregations at the portal area (H & E 100). b): Liver of the rat injected with *Campylobacter coli* showing foci of hepatic necrosis and inflammatory cell aggregation mainly neutrophiles and plasma cells at the portal area (H & E 100)

Table 1: the level of rat Heat Shock Protein 70 (HSP70) at the first and second week post I/P injection of *Campylobacter* species using ELISA

Campylobacter infected groups	Mean of the first week	Mean of the second week
J1 (*jejuni*)	0.72	0.57
J2	0.64	0.63
J3	0.65	0.61
J4	0.67	0.61
J5*	0.7	0.65
Mean level of *C. jejuni* infected groups	0.68	0.61
C6 (*coli*)	0.89	0.83
C7	0.83	0.75
C8	0.78	0.74
C9	0.84	0.76
C10*	0.85	0.81
Mean level of *C. coli* infected groups	0.84	0.78
Control uninfected group	0.2	0.22

*** CJ 5: *Campylobacter jejuni* standard strain (ATCC 33291);*CC10: *Campylobacter coli* standard strain (ATCC 33559).**

DISCUSSION

On-farms control of *Campylobacter* species would reduce the risk of human exposure to this pathogen and have a significant impact on food safety and public health (Lin, 2009). One hundred and fifty samples were collected from different ostrich's farms and the zoo. *Campylobacter* species was detected in water, food and fecal samples. Ugboma *et al*. (2013) recorded that, out of 74 water samples collected, 39 (52.70%) were positive for *Campylobacter* species. They added that *Campylobacter jejuni* (58.97%) and *Campylobacter coli* (28.21%) were identified from the analyzed ground water samples. The ability of *C. jejuni* to survive in water is well recognized by Pearson *et al*. (1996) who recorded that campylobacters can be isolated from the water lines and reservoirs of broiler houses. Contaminated water has been demonstrated to be a source of infection for flocks (Rollins, 1991). Open water receptacles, including troughs and suspended drinkers, contribute to intra flock dissemination of *Campylobacter jejuni* infection

(Smitherman *et al*., 1984). It is widely accepted that feed and feed additives, are not potential sources of infection (Berndtson *et al*., 1996). Through the study we examined 12 food samples with 3 positive *Campylobacter* isolates which was detected from the zoo and private farms. Isolation of *Campylobacter jejuni* from feed in pans and troughs within a house has been documented (Lindblom *et al*., 1986). Twenty one isolates out of 102 fecal samples collected from apparently healthy ostriches were positive for *Campylobacter* species and the highest rate was at the zoo (30.77%) followed by private farms (18.97%) then governmental farms (11.11%). The percentage is closely matched with Ghane *et al*. (2011) who recorded 28 /140 (20%) and 37/120 (30.8%) fecal samples were positive for *Campylobacter* species in Tonekabon and Shiraz respectively. A lower isolation rate was recorded by Marinou *et al*. (2012) from poultry fecal samples (16 out of 830, 1.93%).

Lauková *et al*. (2015) recorded that the examined ostriches were free of *Salmonella* and *Campylobacter* cells.

Cuomo *et al*. (2007) isolated *Campylobacter jejuni* and *Campylobacter coli* from apparently healthy ostriches which considered, theoretically, as potential *Campylobacter* carriers. Simango (2013) recorded that *Campylobacter jejuni* was the most common *Campylobacter* isolated from chicken feces followed by *Campylobacter coli*. It is cleared that a higher incidence rate of *Campylobacter* species was obtained during the warmer seasons than the cold one. Kovats *et al.* (2005) confirmed that the peak incidence of campylobacteriosis observed during late spring and early summer months. Previously, Evans (1997) recorded that there is a higher rate of campylobacters infection in summer than in winter, the reason for these seasonal variations may reflect levels of environmental contamination. Among the experimental infected rats, the liver samples revealed necrotic foci, and edema and paleness of the rat intestine after infection with *Campylobacter* species in agreement with Crawshaw and Young (2003) and Power (2005).

The main goal of the present study was to estimate serum HSP70 levels among the rats experimentally infected with *C. jejuni* and *C. coli* using a commercially available enzyme-linked immunosorbent assay method. De and Roach (2004) developed a sensitive and reproducible ELISA for quantification of soluble HSP27 levels in biological fluid such as serum. HSP70 is involved in inter individual variation in the serum concentration of HSP70 precludes

the use of serum HSP70 levels to distinguish patients from healthy subjects (Njemini *et al*., 2003).

In the present study, a significant difference in HSP70 readings between *Campylobacter jejuni* and *coli* infected groups and the negative control group in the first and second week of infection was recorded. Sera from rats with campylobacter infection had a higher level of HSP70 than those without infection. Extensive studies in the past 10 years suggest that pre-induction of HSP70 may provide therapeutic strategies for Gram-negative sepsis-induced organ or tissue injury in various mammals including humans (Paidas *et al.,* 2002). Bacterial exposure elevates HSP70 which facilitates the recovery from bacterial infection (Kustanova *et al.,* 2006). It is clear that HSP70 is present in low concentrations in uninfected rat. HSP70 presents in human serum after stress and under normal physiological conditions (Hunter-Lavin *et al.,* 2004). HSPs are involved in all important processes associated with growth, such as segmentation, DNA synthesis, transcription, translation, and protein rolling and their transport, through membranes (Tkáčová and Angelovičová, 2012).

CONCLUSION

We concluded that ostrich may be a potential source of *Campylobacter* infection in ostriches. Drinking water and food may be responsible for transmission of *Campylobacter* species. So, various strategies must be applied to prevent the flock from becoming infected with campylobacters through: preventive medical programs especially in the zoo, periodic fecal examinations and treatments for diseased ostrich, good nutrition and potential health care. Serum levels of HSP70 detected by ELISA can be considered as a bio-marker of exposed ostrich to source of *Campylobacter jejuni* and *coli* contamination. Further study is needed to supports the direct relationship between this protein and campylobacteriosis**.**

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Zafar Mahmood from Graduate School of life Sciences, University of Wuerzburg, Germany for his scientific input, experimental planning and revising the manuscript critically for important intellectual content.

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