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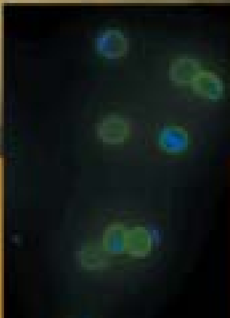
A Study of Cryptosporidium Parvum in Beef



The National Food Centre

RESEARCH & TRAINING FOR THE FOOD INDUSTRY

RESEARCH REPORT NO 62

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A STUDY OF *CRYPTOSPORIDIUM PARVUM* IN BEEF

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SUMMARY

There is increasing concern that foods, particularly those of animal origin, may play a role in the transmission of *Cryptosporidium parvum* to humans. Studies were undertaken to examine the risk posed by C. *parvum* in the beef chain

A novel method was developed for the isolation and detection of C. *parvum* from beef. A key feature of the isolation method was the use of a pulsifier machine (a newly developed instrument that is based on a combined shock wave generator/stirrer that drives microorganisms attached to the sample into suspension without crushing the sample) followed by filtration and centrifugation. Detection was achieved by immunofluoresence using fluorescein isothiocyante (FITC) labelled antibodies. The new method was subsequently used in studies to assess the prevalence and survival of C. *parvum* in the beef chain.

At a commercial abattoir, the prevalence of *Cryptosporidium* oocysts was examined in the faeces of cattle immediately post-slaughter (n=288) and on carcasses following evisceration (n=288). *Cryptosporidium* species were isolated from 21/288 (7.3%) faecal samples at a level of 25 - 37,500 per gram. The parasite was not however detected on carcass meat. The prevalence of *Cryptosporidium* oocysts in water used to wash beef carcasses was also determined at an abattoir with a bore-hole supply (n=46) and a further abattoir with a river water supply (n=49). Both supplies were chlorinated and the river water supply was additionally treated by slow sand filtration. Each water sample (50 l) was collected at the point of application to the carcass. *Cryptosporidium* species were not isolated from bore hole water but were detected in 12/49 river water samples at a level of 0.08 to 9.0 oocysts per litre. These studies show that in a beef abattoir, both faeces and water can be sources of transmission for *Cryptosporidium*.

Experiments were undertaken to determine the survival of C. *parvum* inoculated onto lean and fat beef trimmings that were boxed, frozen and thawed under commercial conditions prior to burger production. The viability of oocysts was determined before and after freeze/thaw treatment. After the freeze/tempering process, 9.46 % of the inoculum were still viable on lean trimmings and 7.17% of the inoculum was still viable on fat trimmings.

The survival of C. *parvum* oocysts on beef following thermal treatments at 60, 75 and 82°C was assessed with a view to establishing the effect of a hot water carcass washing procedure in beef processing. The viability of oocysts was determined before and after heat treatment. The results showed that following treatment at 60°C, the oocyst viability dropped to 83.5% after 15 s and to 64.2% after 1 min. At 75°C, 11.2% of oocysts were viable after treatment for 1 minute. At the highest heat treatment investigated (82°C) only 17.9% of population survived after 15 s exposure, with complete inactivation after 1 min at this temperature.

INTRODUCTION

Cryptosporidium parvum is an emerging pathogen of considerable concern to the water and food industry. *Cryptosporidium* species are protozoan pathogens found in the intestinal tract of animals including cattle and sheep. The parasite is excreted in stable form as an oocyst from the infected hosts which can contaminate the environment and enter the water and food chain. The clinical symptoms of C. *parvum* infection include acute watery diarrhoea with abdominal pain, accompanied by vomiting and weight loss. The disease is usually self-limiting with a duration of 2-3 weeks, although it can last up to 6 weeks. However, in immuno-compromised individuals the illness can become chronic and persistent and may result in the death of the infected person. The infectious dose is low with a mean probability of infection of about 35% after ingesting 100 viable oocysts and a mean probability of infection of about 5% from ingesting 10 viable oocysts.

There have been a number of documented outbreaks of cryptosporidiosis attributed to potable water, well water, spring water and surface water (lakes, rivers, streams). Several outbreaks have also been associated with swimming in contaminated water. If C. *parvum* is present in water, physical treatments such as filtration are necessary to remove the parasite as C. *parvum* is resistant to chlorination.

Recently, food has been identified as a possible source of C. *parvum* with outbreaks of cryptosporidiosis linked to dairy products, apple cider, sausages,

chicken salad and tripe. A lack of suitable methods has limited research and epidemiological studies on the parasite in the food chain. Subjects addressed in this report include a novel method developed for isolation and detection of *Cryptosporidium* spp. from beef, the application of this method to study the prevalence and persistence of the parasite in beef and the effect of some common meat processing operations (freezing and thermal treatments) on the survival of *C. parvum*.

ISOLATION AND DETECTION METHOD

Beef carcass meat was obtained by excision of sections (1.0 cm deep by *ca* 400 cm^2) of flank ("lean") or brisket ("fatty") meat from freshly dressed beef carcass being processed at a commercial abattoir in the Dublin area.

Sections of beef (50 cm^2) were divided into duplicate 25 cm^2 sections and inoculated with *C. parvum* oocysts. The beef was placed in Phosphate Buffer Saline Tween 20 (PBST) (50 ml) and placed in a Pulsifier (Microgen Bioproducts, Camberley, UK) for 30 s. The pulsifier is a newly developed instrument which uses a combined shock wave generator/stirrer to displace microorganisms attached to a sample into suspension without crushing or breaking down the food tissue.

The diluent from the pulsified beef was filtered through a cellulose nitrate membrane (47 mm diameter, 3.0 µm pore size) under vacuum. Following filtration, the membrane was transferred to a sterile 30 ml container with 10 ml of PBST, scraped using an inoculation loop and vortexed for 60 s. The membrane was removed using a sterile forceps, the container was centrifuged (5403 Eppendorf Centrifuge, Germany) for 15 min at 2500 g using a swing out bucket centrifuge and allowed to coast to a natural stop. The supernatant was removed and the pellet resuspended in 100 µl of PBST.

Aliquots (20 µl) of the resuspended pellet were placed at the centre of an 8 or 9 mm well glass slide (Dynal A.S., Norway), air dried, fixed with 50 µl of absolute methanol and further dried in air for approximately 5 min. The fixed suspension was overlaid with 50 µl of fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* monoclonal antibody (Microgen

Bioproducts, UK) and incubated under humid conditions for 1 h at 37°C. A drop of glycerol (50 %) was placed on the well of each slide, a coverslip was applied and the slide was sealed. The contents in the well of the slide were examined using an epifluorescent microscope (Olympus, BX61, Japan) (600 x magnification; excitation wavelength 495 nm; emission wavelength 519 nm). The concentration of oocysts in each suspension was determined from at least 10 replicate counts.

The recovery of C. *parvum* from the surface of "lean" or "fatty" beef pieces is presented in Table 1. Higher numbers of oocysts ($P < 0.05$) were recovered from lean beef at all three inoculation levels. This may be related to differences in oocyst binding avidity in the two beef tissue types examined. Alternatively, the differences may be related to how the lean and fatty tissues behave during sample processing. The recoveries achieved are in line with other parasite detection methods reported in the literature.

The developed method will be of value in establishing the incidence of C. *parvum* on beef and in future research on this parasite.

Table 1. Number of *C. parvum* recovered from lean and fatty beef surfaces using the newly-developed isolation and detection technique.

Meat type	Inoculum (mean no.)	Oocysts recovered	
		Number	%
Lean	38560	49507	128.4[a]
Fat	38560	32945	85.4[b]
Lean	512	299	58.4[c]
Fat	512	222	43.4[d]
Lean	105	39	37.1[e]
Fat	105	15	14.3[f]

[1] Means with the same superscripted letters do not differ significantly ($P < 0.05$).

PREVALENCE AND NUMBERS IN THE BEEF CHAIN

The prevalence and numbers of C. *parvum* in animal faeces, beef carcasses and in water used to wash carcasses was assessed at commercial beef abattoirs.

Faeces and carcasses

Faecal (n=288) and carcass (n=288) samples were collected at a single commercial beef abattoir during a one-year period from February 2002 (Qtr1: Feb-April, Qtr 2: May-July, Qtr3: Aug-Oct and Qtr 4: Nov –Jan 2003). The factory was visited 3 times each quarter and on each visit, 24 cattle (12-36 mths old) were tagged at the beginning of the slaughter process and faecal and carcass samples were then collected from each tagged animal.

The faeces was collected from the rectum of animals immediately post-slaughter. Following hide removal, a beef sample (25 cm^2) was excised aseptically from the rump and following evisceration, a sample was excised from the brisket.

Faecal samples (1.0g) were examined for *Cryptosporidium* using a salt flotation technique while the method described above was used to isolate and detect *Cryptosporidium* from the beef samples.

Cryptosporidium species were isolated from 21/288 (7.3%) of faecal samples at concentrations ranging from 25 to 37,500 per gram. There was a higher prevalence of the parasite during spring and winter than in summer and autumn ($p < 0.001$) (Table 2)

Following molecular examination of a selection of the positive samples (11/21) by the polymerase chain reaction (PCR) and sequence analysis, 5/11 (45.5%) were found to be C. *parvum* genotype 2 and 6/11 (54.5%) were C. *andersoni*. In general, C. *andersoni* is not linked to human illness.

Although *Cryptosporidium* was detected in the faeces, it was not detected on any of the carcass samples. This may be related to the fact that the number of oocysts was low in the majority of faecal samples (12/21 had counts < 100 oocysts per g) which may have precluded transmission onto the carcass. Studies conducted on bacteria suggest that the transmission rate from faeces to carcass may be as low as 1 in 10,0000 organisms. The negative results may

Table 2. The prevalence of *Cryptosporidium* species in the faeces of slaughtered cattle during each season.

		Positive		
Season	No. sampled	Number	%	Range (oocysts/ g faeces)
Spring	72	4	5.5	100 - 37500
Summer	72	7	9.7	25 - 50
Autumn	72	3	4.1	50 - 75
Winter	72	7	9.7	75 - 1000
Total	**288**	**21**	**7.3**	**25 - 37500**

also have been partly attributable to a high variability in the number of oocysts which the method would have recovered, with as few as 14.3 % of oocysts recovered from a sample with very low numbers of the parasite present (see Table 1). Alternatively, it may reflect a low adhesion of oocysts to beef tissue but further research would be necessary to establish this.

Water used to wash carcasses

During a one-year period from January to December 2002, water samples were collected at the point of application to the carcass from an abattoir with a river water supply (n=50) and from a further abattoir with a bore-hole water supply (n=46). Each of the water supplies was chlorinated while the river water supply was also subjected to slow sand filtration.

From each abattoir, a 50 litre water sample was collected on each sampling occasion at the point of application to the carcass. Each 50 l sample was filtered through a cellulose nitrate membrane filter (142mm diameter, 1.2μm pore size). The membrane was placed in a sterile stomacher bag with 50ml of PBST and was scraped to remove any adhering *Cryptosporidium* oocysts. The resulting suspension was centrifuged at 2500g for 15 min using a swing out

rotor with no brake applied during deceleration. The supernatant was aspirated, the pellet was resuspended in 10ml of water and transferred to a sterile Leighton tube. Immunomagnetic separation (IMS) was performed using beads coated with anti-*Cryptosporidium* monoclonal antibodies (Immucell Corporation, Portland, USA). Following IMS, the sample was mounted on a slide, labelled and examined by immunofluorescence as described above.

Cryptosporidium species were isolated from 12/49 (24.5 %) river water samples but were not detected in bore-hole water samples. The concentration of oocysts in positive water samples ranged from 0.08 to 9 oocysts per litre. *Cryptosporidium* species were isolated from river water samples during each season with the highest prevalence during summer.

It is clear that the treatment procedures (chlorination and slow sand filtration) for the river water supply were insufficient to remove *Cryptosporidium* oocysts. While *Cryptosporidium* spp are resistant to chlorine, slow sand filtration is generally regarded as an efficient method to remove *Cryptosporidium* oocysts from water. The presence of oocysts in the treated water, therefore indicates that the concentration of oocysts in the raw

Table 3. Incidence of *C. parvum* oocysts in abattoir water samples (50 litre samples) collected from a river water source over a 1 year period.

Season	No. sampled	Positive		Range (per litre)
		Number	%	
Feb-April	5	1	20.0	0.02
May-July	14	5	35.7	0.04 - 2
Aug-Oct	15	4	26.6	0.1 - 0.4
Nov-Jan	15	2	13.3	0.08 - 9
Total	**49**	**12**	**24.5**	**0.08 - 9**

(untreated) river water was probably very high and also that the slow sand filtration system may not have been optimised for oocyst removal. The size of sand grains has been found to significantly affect oocyst removal with an inverse relationship between grain size and removal efficiency.

The study has demonstrated that *Cryptosporidium* spp. can enter a beef abattoir in the water supply and is a potential source of carcass contamination. It highlights the need for assessment and optimisation of the efficiency of treatment systems for water which is used in food production. It also emphasises that the quality of raw water source directly influences the level of treatment required and as such a protected bore water supply is preferable to a surface water supply.

EFFECT OF FREEZING AND THAWING

The survival of C. *parvum* was estimated by inoculation of the parasite onto lean or fat beef trimmings that were boxed, frozen and thawed under commercial conditions prior to burger production.

On three separate visits to a beef abattoir, a box of beef trimmings (28 kg on average) was obtained. Each box contained an average of 30 pieces of beef trimmings each of which had an approximate surface area of 230 cm^2 (8-15 cm x 15-25 cm) and weight of 575 g (450-700 g). Beef trimmings that were visually lean or visually fat were chosen from the box and classified as lean and fat beef trimmings respectively.

At the laboratory, a delineated area of the trimmings was inoculated with 250,000 oocysts, and allowed to dry at room temperature (below 20°C) for 15 min.

The inoculated beef trimmings were placed among the other pieces of uninoculated chilled beef trimmings in the original box of trimmings, at three positions: top, centre and bottom. The subsequent temperature profile of the trimmings was recorded every 2 h using a temperature data logger during the normal commercial processes of blast freezing to -20°C ± 5°C (60 h) and storage at this temperature for 21 d and normal commercial tempering

conditions (-3°C for 45 h). The thawed trimmings were examined for the presence of *Cryptosporidium* using the above method in conjunction with a viability assay (DAPI/PI vital dye assay).

The temperature profile data from the freeze/thaw cycle showed a slower rate of freezing in the middle of the box (-1.5°C after 48h) than at the top (-8.9°C after 48h) and bottom (-11.6°C after 48h) and almost 72h were required for the box to reach the desired temperature of approximately –20°C.

After this freeze/tempering process, 9.46 % of the inoculum was still viable on lean trimmings and 7.17% of the inoculum was still viable on fat trimmings. When the viability of the population of recovered oocysts from lean beef trimmings was compared relative to their position within the box, no significant difference was noted. Also no significant interaction was noted on the viability of the oocyst population recovered from the fat beef trimmings relative to the position they occupied within the box during processing.

The results show that freezing / tempering will not inactivate all oocysts present on meat. However, current commercial processing can lead to the inactivation of very significant proportions of a contaminating oocyst population. Such processing, if carefully and rigorously applied within HACCP and related schemes, can provide useful and highly desirable reductions in the numbers of viable oocysts present on contaminated trimmings and derived products.

EFFECT OF THERMAL TREATMENTS

Beef carcasses are often washed with water before chilling to removal visible dirt. Depending on the temperature of the water used it may also have an anti-microbial effect. In this study, the survival of C. *parvum* oocysts on lean beef (knuckle) following thermal treatments at 60, 75 and 82°C and a control of 25°C was assessed with a view to establishing the effect of a hot water carcass washing procedure on survival of oocysts. Meat pieces (72.5cm^2) were inoculated with 250,000 oocysts and then vacuum-packed.

The sealed bags were immersed in a water bath that was adjusted to the desired temperatures for treatment (60, 75 and 82°C) or control (25°C). At intervals of 15, 20, 30, 45 and 60 s, samples were removed from the water bath and examined for the presence of C. *parvum* using the method described above in addition to a viability staining assay.

The results (Table 4) indicate that, at 60°C, oocyst viability dropped gradually from 83.5% after 15 s to 64.2% after 1 min. After 15 s at 75°C there was a large loss in viability (53.7%) which continued to decrease, reaching 11.2% after treatment for 1 min. The final treatment of 82°C proved highly effective for inactivating oocysts with a survival of only 17.9% after 15 sec, dropping to zero survival following treatment for 1 minute. As expected, the control treatment of 25°C for 60 s did not significantly reduce the viability of the oocysts (97.8% viable).

These results can be used when establishing appropriate cooking times and temperatures for meat products and on appropriate water temperature and contact times for the washing of beef carcasses following dressing. The results from this study indicate that adequate cooking times will inactivate C. *parvum*. The parasite is less resistant than most food poisoning bacteria so

Table 4. Oocyst viability (%) following treatment of beef at four different temperatures (control, 60, 70 and 82°C).

		Temperature (°C)		
Time (s)	Control (25°C)	60	75	82
15	97.8	83.5	53.7	17.9
20	97.8	77.2	45.9	10.8
30	97.8	74.7	37.2	4.6
45	97.8	71.6	25.3	2.2
60	97.8	64.2	11.2	0

industrial processes such as pasteurisation and cooking, which are in place to inactivate bacteria, are sufficient to inactivate this parasite.

CONCLUSIONS

- A method was developed for the isolation and detection of C. *parvum* in beef, which can be used in studies on the prevalence and persistence of the pathogen in food.
- *Cryptosporidium* is present in the faeces of a small percentage of cattle presented for slaughter (7.3%) at a level of 25 - 37,500 oocysts per g.
- The parasite was not detected on any carcass meat samples (n=288) examined over a period of 1 year.
- In water used to wash beef carcasses, *Cryptosporidium* species were not isolated from water sourced from bore holes but were detected in 12/49 water samples sourced from a surface water supply (river) at a level of 0.08 – 9 oocysts per litre.
- Commercial freezing and thawing of beef trimmings was shown to considerably reduce the viability of C. *parvum* but a small percentage of the oocysts remained viable on lean trimmings (9.46 %) and on fat trimmings (7.17%).
- Thermal treatment at 60°C reduced oocyst viability to 83.5% after 15 s and to 64.2% after 1 min. At 75°C, 11.2% of the population remained viable after treatment for 1 minute. At the highest heat treatment investigated (82°C), only 17.9% of population survived 15 s exposure, with complete inactivation after 1 min at this temperature.

RECOMMENDATIONS TO INDUSTRY

This is the first study on the role of beef in the transmission of *Cryptosporidium* to humans. The risk posed by *Cryptosporidium* on beef is low as in general these products are cooked; this should effectively inactivate any oocysts present. However, under-cooking or cross-contamination from raw to ready-to-eat foods is a risk, particularly because of the low infective dose of this organism. C. *parvum* may enter the abattoir either via contaminated faeces or surface water used to wash carcasses. However, in this study there was no detectable transmission of the pathogen to the beef carcass. If the parasite is present on meat, processing steps including thermal treatments substantially reduce the viability of the parasite.

The most effective control steps are to ensure the water used in food processing is assessed to ensure that sufficient physical treatments (ie coagulation, flotation, filtration) are in place to remove oocysts from the raw water supply. In addition, HACCP (Hazard analysis critical control point systems) (Bolton *et. al.*, 2000) should address parasites as a potential hazard in the meat chain.

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