Curr Microbiol (2010) 60:38–41
DOI 10.1007/s00284-009-9497-5

Viability of *Rhodococcus equi* and *Parascaris equorum* Eggs Exposed to High Temperatures

Laurent Hébert · Julien Cauchard · Pauline Doligez · Lola Quitard · Claire Laugier · Sandrine Petry

Received: 20 July 2009 / Accepted: 24 August 2009 / Published online: 1 September 2009

Abstract There is great concern about the potential pathogen contamination of horse manure compost spread in the same fields horses graze in. To ensure that pathogen destruction occurs, temperatures need to be sufficiently high during composting. Here, we investigated the survival rate of two marker organisms, *Rhodococcus equi* and *Parascaris equorum* eggs, exposed to temperatures potentially encountered during horse manure composting. Our results show that the time required to achieve a 1 log10 reduction in *R. equi* population (*D*-value) are 17.1 h (±1.47) at 45°C, 8.6 h (±0.28) at 50°C, 2.9 h (±0.04) at 55°C and 0.7 h (±0.04) at 60°C. For *P. equorum* eggs we show that at 45 and 50°C, 2 log10 reduction of viability is reached between 8 and 24 h of incubation and that it takes less than 2 h at 55 and 60°C to achieve a viability reduction of 2 log10. These results are useful for identifying composting conditions that will reduce the risk of environmental contamination by *R. equi* and *P. equorum* eggs.

Introduction

During the last decades, composting has become the most common technological alternative for manure valorization although such practices can lead to increased contamination of the farm environment and may pose an infection risk to humans or animals [14]. This problem of potential pathogen contamination of agricultural products with human and animal pathogens present in manures and composts [8, 12] is especially true in horse farm where compost can be spread in the same fields horses graze.

Although the mechanism for pathogen inactivation during composting is complex and not solely dependent on temperature and time [19], temperature is generally considered as the main factor for sanitation. Here, we aimed to determine heat inactivation temperature–time relationships to ensure health security of horse manure compost. As survival indicator we chose two horse pathogens with fecal–oral life cycle known to play an important role in contamination of the soil environment of equine farms and generally recognized as very resistant to environmental conditions: *Rhodococcus equi* and *Parascaris equorum* [2, 4].

R. equi is the causative agent of chronic suppurative bronchopneumonia, and ulcerative enteritis associated with a high mortality rate. It is a facultative intracellular pathogen affecting foals up to 6 months old, considered as one of the most significant pathogens in the equine breeding industry [18]. *R. equi* is also an opportunistic zoonotic pathogen causing cavitary pneumonia predominantly in immunocompromised humans, particularly in AIDS patients [9]. The route of infection of foals is generally considered to be inhalation of aerosolized bacteria from soil contaminated by equine feces [9]. *R. equi* is able to survive and multiply in manure, soil, and gastrointestinal tract and fecal concentrations can be as high as 10^8 *R. equi*/g feces in foals with *R. equi* pneumonia [17].

For *P. equorum*, both migrating larvae and adult worms can cause clinical signs in suckling and weanling foals.

L. Hébert (✉) · J. Cauchard · C. Laugier · S. Petry
AFSSA, Laboratoire d'Etudes et de Recherches en Pathologie Equine, IPC, Goustranville, 14430 Dozulé, France
e-mail: l.hebert@afssa.fr

P. Doligez
Les Haras Nationaux, Direction des Connaissances, 61310 Le Pin au Haras, France

L. Quitard
Pôle de compétitivité Filière équine, 23 rue Pasteur, 14120 Mondeville, France

Respiratory form coincides with the migration phase and includes inappetence, nasal discharge, and coughing. Adult worms may cause unthriftiness, reduced growth rate with an enlarged abdomen, and rough coat. Colic, diarrhoea, small intestinal intussuception, impaction, or rupture are occasionally observed [3]. Ascarids are especially dangerous for foals up to 6 months old; adult horses commonly are immune to the infection [4]. The major route of transmission is ingestion of embryonated eggs in the environment [15]. The female of *P. equorum* lay up to 200,000 eggs per day, these eggs are passed to the outside in the manure and become infective in the grass [4]. In a single day, a foal can contaminate the environment with millions of eggs [5] which are very resistant to environmental conditions and may remain infective for years on pastures, and in stalls.

The objective of this research was to determine under laboratory conditions, time–temperature relationships that must be obtain in windrow to prevent any environmental contamination with *R. equi* and *P. equorum* eggs.

Materials and Methods

Bacterial Strains, and Culture Conditions

The bacterial strain used in experiments was *R. equi* ATCC 33701, a virulence plasmid-bearing strain. It was grown on Brain Heart Infusion (BHI) medium at 37°C with shaking at 200 rpm.

Collection of *P. equorum* Eggs

Adult females *P. equorum* were collected from the intestine of an immunodeficient 14-year-old mare dead from septicaemia. Suspensions of *P. equorum* eggs were prepared as describe by Koudela and Bodeček [13]. We obtained a working egg solution with approximately 33,000 eggs/ml. *P. equorum* eggs were stored at 4°C in sterile distilled water with 10 μg/ml gentamicin sulfate to prevent further bacterial contamination.

High Temperature Treatment and Counting of *R. equi*

The strain was cultivated in BHI (100 ml). Once the stationary phase was reached (after 16 h at 37°C), 20 ml of culture was divided in five 50-ml Falcon tube (BD, Franklin Lakes, NJ, USA) and each tube was incubated in thermo-regulated ovens at tested temperatures (37, 45, 50, 55, and 60°C). Sample aliquots of 0.1 ml were removed from each culture for plating at the specified times during the course of the experiment. Tenfold serial dilutions in 0.9% NaCl were plated on BHI agar at each time point and Colony-Forming Units (CFU) were enumerated after 48 h of incubation at 37°C by counting two plates at two different dilutions. All experiments were performed in triplicate.

D-values were determined from the linear portion of the survivor plots using linear regression analysis. *D*-values are defined as the time required to achieve a 1 log10 reduction in the bacterial population at a designated temperature.

High Temperature Treatment and Control of *P. equorum* Eggs Survival

Sample containers with approximately 50,000 eggs of *P. equorum* in 10 ml of distilled water were incubated in thermo-regulated ovens at tested temperatures (45, 50, 55, and 60°C). Sample aliquots of 2,000 eggs were removed from each sample container at the specified times during the course of the experiment and incubated for 4 weeks at 27°C and survival of eggs were checked. Briefly, the development of eggs was evaluated under a microscope: eggs that had developed to the fully larvated stage were considered viable, while all others were deemed inactivated [13]. Survival was defined as the ratio of viable eggs present after the challenge treatment to viable eggs at time zero. For each time point ~600 eggs were counted. All experiments were performed in triplicate.

Results

Sensitivity of *R. equi* to Heat Treatment

We examined the survival of *R. equi* strain ATCC33701 in the presence of temperatures potentially encountered in windrows. We monitored the survival of the bacteria over 6 days of incubation in BHI medium at 37, 45, 50, 55, and 60°C. The viability of *R. equi* was not affected by 6 days of incubation at the control temperature of 37°C. At 45, 50, 55, and 60°C *R. equi* population dropped below detectable levels after 6, 3, 1 days, and 8 h of incubation, respectively (Fig. 1). Our results show that *R. equi* viability decrease is log-linear. Determined *D*-value are 17.1 h (±1.47) at 45°C, 8.6 h (±0.28) at 50°C, 2.9 h (±0.04) at 55°C, and 0.7 h (±0.04) at 60°C.

Sensitivity of *P. equorum* Eggs to Heat Treatment

Results given in Fig. 2 show that at 45 and 50°C the course reduction of *P. equorum* eggs viability over time is almost identical: 2 log10 reduction is reached in less than 24 h of incubation. At 55 and 60°C it takes less than 2 h to achieve a 2 log10 reduction. These results are consistent with those of Koudela and Bodeček [13] who described that 5 min of

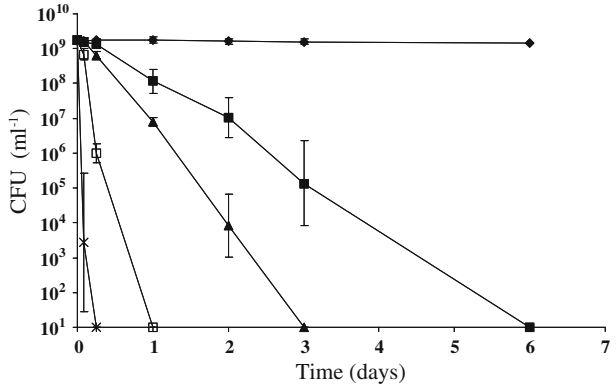

Fig. 1 Effect of high temperature on *R. equi* survival versus time. Each value represents the mean CFU/ml (±SD) from three independent experiments. Symbols: *filled diamond*, 37°C; *filled square*, 45°C; *filled triangle*, 50°C; *open square*, 55°C; *times*, 60°C

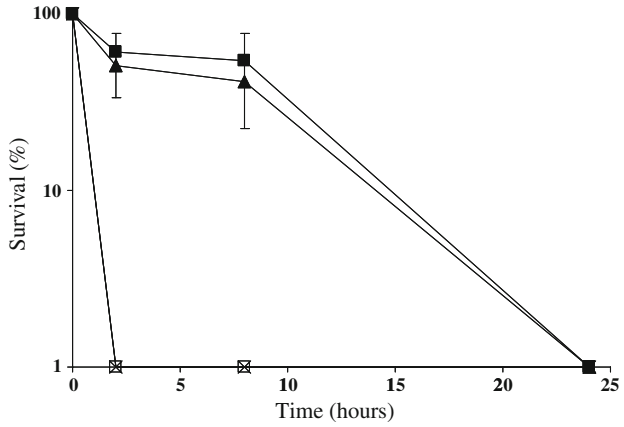

Fig. 2 Effect of high temperature on *P. equorum* eggs viability versus time. The values shown are means ± SD of the of *P. equorum* eggs viability percent from three independent experiments. Symbols: *filled square*, 45°C; *filled triangle*, 50°C; *open square*, 55°C; *times*, 60°C

incubation at 60°C is sufficient to induce a viability loss of *P. equorum* eggs of more than 99%. Due to the rapid decrease of *P. equorum* eggs viability, *D*-values were not determined because we felt that the amount of data was insufficient to obtain a representative regression.

Discussion

Horse manure composting must be closely control to prevent environmental contamination which can induce detrimental effects on foals but also have public health consequences. For example, it has been shown that the infection of a gardener by *Streptococcus equi* subspecies *zooepidemicus* appears to have been acquired through horse manure manipulation [14]. Moreover, it has previously been shown that a *Listeria monocytogenes* thermotolerant variant induced by sublethal heat treatment or by selection of heat resistant cells could have caused outbreaks [1].

R. equi and *P. equorum* eggs are very resistant to environmental conditions and may remain infective for years on pastures and in compost. It is known that *R. equi* can grow up to 40°C [10] but, to our knowledge, there is no data describing *R. equi* survival towards higher temperatures. For *P. equorum* eggs we found only one study in the literature, this study determined survival of eggs exposed to high and low temperatures during short time (1–5 min) [13]. Thus, in the context of pathogens control during horse manure composting we decided to study long-term survival of *R. equi* and *P. equorum* eggs towards high temperatures.

By considering that *R. equi* fecal concentration can be as high as 10^8/g in infected foals [17], our results show that, to reduce the number of viable bacteria below detectable level, manure must be approximately maintained at a minimum temperature of 45°C for 5.7 days, 50°C for 2.9 days, 55°C for 24 h, and 60°C for 5.7 h.

Given that the highest fecal *P. equorum* egg output in experimentally infected foal is 50 million per day, we can deduce that it seems necessary to reach temperatures greater than or equal to 55°C on a relatively short period (at least 8 h) or temperatures between 45 and 55°C during several days (more than two days) to reduce the number of viable eggs below detectable level.

The United States Environmental Protection Agency's publication [7] on control of pathogens in biosolids describe two standard requirements for composting, either a "Process to Significantly Reduce Pathogens" (PSRP standard) or a "Process to Further Reduce Pathogens" (PFRP standard). To meet PFRP requirements, composting operation must ensure that the process last for 15 days or longer at a temperature greater than 55°C (and windrow piles must be turned five times during this period), to meet PSRP requirements the compost pile must be maintained at a minimum of 40°C for at least five days and during the five-day period, the temperature must rise above 55°C for at least 4 h. By comparing with EPA requirements, our results show that PFRP is sufficient to obtain a thermal destruction of two major horse pathogens: *R. equi* and *P. equorum* eggs.

These results are useful for identifying composting conditions that will reduce the risk of environmental contamination by *R. equi* and *P. equorum* eggs; however, other variables should be considered when interpreting these values since it is observed that levels of heat resistance can be influenced by factors as tested strain, and culture conditions [6, 16]. Finally, to ensure that in vitro heat survival experiments can be transposed to the natural process of horse manure composting, fields experiments involving for example, the use of sentinel system [11] are still required.

Acknowledgments L. Hébert was founded by a grand awarded by the Fonds EPERON (FNCF Paris, France). We are very grateful to the Unit observatoire anatomo-pathologique et epidémiologique des maladies équines majeures ou émergente (AFSSA Dozulé, France) for providing adult *P. equorum*.

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