Efficacy of amine-based disinfectant KENO™COX on the infectivity of Cryptosporidium parvum oocysts

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ABSTRACT

Cryptosporidium parvum is a zoonotic protozoan parasite that may cause severe neonatal diarrhoea or even mortality in newborn ruminants: its oocysts are extremely resistant to normal environmental conditions and to most common disinfectants. KENO™COX, a patent pending amine-based formula, was tested for its ability to inactivate C. parvum oocysts. The Daugschies assay (2002), a standardized assay for chemical disinfection initially described for Eimeria spp., was adapted for C. parvum oocysts. KENO™COX diluted in water at 2% and 3% concentration and incubated with oocyst suspensions for 2 h, allowed a significant reduction in viability, lysing 89% and 91% of oocysts respectively. Infectivity of the remaining C. parvum oocysts was assessed by inoculation to C57 Bl/6 neonatal mice. Each mouse received 2.5 μl of a suspension initially containing 500,000 oocysts before contact with KENO™COX. Six days post inoculation, the intestinal parasite load was significantly reduced by 97.5% with KENO™COX 2% compared to that of the mice inoculated with untreated parasites. KENO™COX 3% completely eliminated infectivity of oocysts. The number of oocysts remaining infectious in the inoculum treated with KENO™COX 2% was calculated from an inoculated dose–response curve: it was estimated at about 48.6 oocysts among the 500,000 oocysts initially treated corresponding to 99.99% of inhibition. These results demonstrate the high efficacy of KENO™COX against C. parvum oocysts. Combined with an appropriate method of cleaning, the application of KENO™COX may be a useful tool to reduce cryptosporidial infectious load on farm level.

1. Introduction

Cryptosporidium parvum is a ubiquitous intestinal protozoan parasite, which can infect a wide range of mammalian host species, including humans. This pathogen is responsible for enteric infection and debilitating diarrhoea or even mortality in newborn ruminants (calves, kids, lambs) within the first two weeks of life (Morin et al., 1976; Fayer and Ungar, 1986; Naciri et al., 1999). Due to intensive farming methods, cryptosporidiosis became an important pathology in cattle (Lefay et al., 2000) that causes significant economic losses. Animals are infected orally, generally within the first hours of life by mother, congener or environmental contact and shed then large numbers of C. parvum oocysts. These oocysts shed into the environment are immediately infectious for other animals or humans. Thus, there is a great need of controlling calf cryptosporidiosis in combining prevention or treatment of animal infection and environmental disinfection. There are no licensed vaccines to prevent infection (Wyatt et al., 2010). The early exposure to C. parvum in first hours or days of the animal life makes calf vaccination difficult. Passive immunization of calves against C. parvum infection provides protection against disease but requires high titres of specific C. parvum antibodies in the intestinal lumen.

during a sufficiently long period (Fayer et al., 1989; Naciri et al., 1994). Cow immunization by one intramuscular injection of C. parvum oocysts followed by 3 injections directly in the mammary gland is an impractical procedure, not suitable in a milk production environment and too expensive. Some drugs showed effective against cryptosporidiosis: halofuginone in lambs or calves (Naciri and Yvoré, 1989; Naciri et al., 1993; Lefay et al., 2001; Joachim et al., 2003b), paromomycin in kids (Mancassola et al., 1995), and decoquinate in kids (Mancassola et al., 1997); however, only halofuginone is approved for use in cattle in France. These active molecules prevent or treat the symptoms and decrease the oocyst excretion without eliminating it, resulting however in a reduction in the total numbers of environmental oocysts, thus reducing the parasite load on the farm.

As other coccidian parasites, C. parvum oocysts are highly resistant to a wide range of environmental conditions. If not exposed to extreme temperatures (freezing, thawing or desiccation), they can remain viable and infectious for several months, either in soil or water; therefore, infection can rapidly spread via the fecal-oral route. Sanitary programs cannot be neglected if C. parvum is to be reduced on farm level. The implementing of a cleaning and disinfecting program has for objective to reduce environmental oocyst loads in order to delay, as long as possible, the first contact between the parasite and the calf and to control transmission between calves.

C. parvum exogenous stage, the oocyst, is resistant to most of the disinfectants at the recommended concentrations commonly used (Robertson et al., 1993). Common water disinfectants as ozone, chlorine-based compounds used to treat drinking water are ineffective against C. parvum (Korich et al., 1990; Fayer, 1995; Weir et al., 2002). Use at concentrations or exposure times that are sufficient to kill C. parvum would present an unacceptable hazard to humans and livestock (O’Donoghue, 1995).

C. parvum oocysts are resistant to sodium hypochlorite, which is used in our laboratory to purify them. Steamed water may be applied at high pressure for several minutes, but a phenomenon of aerosolization of the parasite can spread contamination to surrounding surfaces. However, more recently, several research teams showed a reduction of viability and infectivity of C. parvum oocysts with different disinfectants and different methods such as lysis test, excystation rate and vital-dye assay performed to assess viability of oocysts while combination cell culture and quantitative PCR or oocyst infectivity in an animal model were proved the most performing to reveal the oocyst inactivation. Like this, a 24 h exposure to 0.007 M ammonia decreased significantly the viability of oocysts in in vitro essays (Jenkins et al., 1998), a 4-min exposure to 6% hydrogen peroxide or a 13-min exposure to ammonium hydroxide-amended windshield washer fluid reduced infectivity of C. parvum oocysts in cell culture 1000-fold (Weir et al., 2002), 10% Ox-Virin (hydrogen peroxide plus peracetic acid) for 60 min and 3% Ox-Agua (hydrogen peroxide plus silver nitrate) for 30 min completely eliminated C. parvum oocyst infectivity for mice (Quilez et al., 2005) and 2 cresolic compounds Neopredisan® 135-1 and Aldeco® TGE (4% for 2 h) consistently inactivated more than 99.5% of C. parvum oocysts using cell culture and quantitative real time PCR (Shahiduzzaman et al., 2010).

In this study, KENO™COX, a patent pending amine-based formula was tested to assess its efficacy to inactivate C. parvum oocysts. Destruction and reduction of C. parvum oocyst numbers were evaluated by an in vitro test and infectivity of the remaining oocysts was determined using an inoculation test in C57 Bl/6 neonatal mice.

2. Materials and methods

2.1. Inactivation test on C. parvum oocysts

2.1.1. Preparation of C. parvum oocysts

The inactivation test was carried out with a C. parvum strain initially isolated from the faeces of an infected child (Arnaud-Battandier et al., 1982), and regularly maintained (at least every 6 months) at the INRA laboratory by passage in calves experimentally infected. For the test, a 2 day-old calf was experimentally infected with 107 C. parvum oocysts. The oocysts shed were isolated from calf faeces collected from 4 to 7 days post inoculation by filtration and diethyl ether sedimentation. The pellet containing oocysts was washed three times in distilled water, and the oocysts were counted in a hemacytometer (Thoma cell) using a 0.08% malachite green solution (malachite green 0.08 g, sodium dodecyl sulphate 0.1 g, distilled water 100 ml) before storing at +4 °C in 2.5% potassium dichromate until use. Thoma cell volume being of 0.1 mm3, 1 oocyst counted matches to 10,000 oocysts/ml.

From this pool, 30 × 105 oocysts were washed three times in distilled water (5 min at 1860 g) in order to remove the potassium dichromate solution. The oocysts in the last pellet were suspended in 30 ml of water and distributed in three 35 ml conical glass centrifuge tubes (i.e. 10 ml oocyst suspension containing 106 oocysts/ml in each tube). Each tube was identified, one tube was marked “C” for control and both others were marked “K2 and K3” for KENO™COX at 2 and 3% concentrations. The number of oocysts was counted 6 times in 0.08% malachite green solution on a Thoma cell before exposure to the disinfectant (contact time CT = 0). The initial number was 106 oocysts/ml. The average of the 6 counts was recorded.

2.1.2. Inactivation test

Product tested in this study was KENO™COX, a amine-based disinfectant. Disinfection was initiated in adding 10 ml of a KENO™COX solution freshly prepared at a double concentration in water (4 and 6% KENO™COX) in the conical glass centrifuge tubes K2 and K3 containing 10 ml of oocyst suspension (giving 107 oocysts/tube in total). Ten millilitres of water was added in the conical centrifuge tube C containing 10 ml of oocyst suspension (given 107 oocysts/tube in total). Tubes were put on a stirring plate for the whole contact time (2 h) at room temperature (≈22 °C). At the end of contact time (CT2), tubes (K2, K3 and C) were immediately centrifuged (10 min at 2500 t/min) and the oocysts were washed three times in water to remove the disinfectant. No neutralizing agent was applied therefore, contact time was considered as the time oocysts were in suspension, excluding subsequent
washed. After the last washing, oocysts were suspended in 50 μl of water to obtain the equivalent of 500,000 oocysts in the untreated condition in 2.5 μl for the control tube. At CT2, the total number of oocysts present in each tube was counted 6 times in 0.08% malachite green solution on a Thoma cell and the average of 6 counts was calculated.

The recovery rates in C, K2 and K3 were calculated by using the formula \( \frac{[\text{oocyst number at CT2}] - [\text{oocyst number at CT0}]}{\text{oocyst number at CT0}} \times 100 \). The inactivation percentage or efficacy of the product in destroying the oocysts was then calculated as follows:

\[
\% \text{ efficacy} = 100 - \text{recovery rate or} \left( \frac{\text{oocyst number at CT0} - \text{oocyst number at CT2}}{\text{oocyst number at CT0}} \right) 
\]

CT0 and CT2 = contact times before (0 h) and after (2 h) exposure to the product.

The disinfectant efficacy was corrected to take into account the recovery rate in control C, which was not 100%. Corrected KENO\(^\text{TM}\)COX recovery rate was then calculated as follows: \( \left[ \frac{\text{recovery rate in K}}{\text{recovery rate in C}} \right] \times 100 \) and consequently the corrected KENO\(^\text{TM}\)COX efficacy derived from the difference of the corrected KENO\(^\text{TM}\)COX recovery rate from 100.

The infectivity of the remaining oocysts after 2 h contact time was evaluated by inoculation to C57BL/6 neonatal mice.

2.2. Oocyst infectivity test in C57 Bl/6 neonatal mice

Six litters of 2-day-old neonatal C57BL/6 mice, individually housed with their dams under specific pathogen-free conditions, maintained at constant temperature and humidity with food and water given ad libitum, were used for infectivity tests. Two litters composed of 7–8 pups per litter were used for each group: 2% KENO\(^\text{TM}\)COX-exposed oocysts (K2), 3% KENO\(^\text{TM}\)COX-exposed oocysts (group K3), disinfectant-unexposed oocysts (control group C). Neonate mice were orally inoculated using a calibrated micropipette; each pup received 2.5 μl of an oocyst suspension containing 500,000 \( \text{C. parvum} \) oocysts at the contact time 0 (CT0). In these small animals the level of infection is expressed by the number of oocysts in the intestine as described in Lacroix et al. (2001). Briefly, at peak oocyst excretion, 6 days post-inoculation, all neonatal mice were killed by cervical dislocation. Because infection does not always spread homogeneously along the intestine (Current and Reese, 1986), the entire (small and large) intestine was removed from each mouse individually, placed in 1 ml PBS (pH 7.2) and weighed. Each intestine was crushed using an Ultra-Turrax up to obtaining a homogenous mixture (3 times 10 s). Homogenates were stored at +4 °C for a maximum of 1 day until they were quantified. The number of oocysts present per ml in blinded homogenates was counted in Sheather’s solution on a Thoma cell and the total number of oocysts present in each intestine, parasite load or oocyst excretion (OE) was determined taking into account the homogenate weight. The oocyst excretion in the neonatal mouse intestine of the KENO\(^\text{TM}\)COX groups was compared to that of control group C. The percentage reduction compared to C was calculated.

2.3. Inoculated dose–response study

An inoculated dose–response study was conducted to determine by linear regression the ingested dose corresponding to the intestinal oocyst excretion measured in the KENO\(^\text{TM}\)COX groups. Four litters (8 and 9 pups/litter) of 2-day-old neonatal C57Bl/6 mice were used to study the intestinal oocyst excretion according to the unexposed control oocyst dosage. The doses of 5000, 500, 250 and 100 oocysts per neonatal mouse were tested.

As previously described, at 6 days post inoculation, oocysts excreted in the entire intestine were counted using a Thoma cell. From mean total oocyst excretion for each dose-group, a regression line and a correlation coefficient \( R^2 \) were calculated according to Daugschies et al. (2002). The number of oocysts remaining infectious after disinfection (infectious dose \( \text{ID} \)) was estimated from this line according to the following formula:

\[
x = \frac{y - a}{b},
\]

where \( x \) is the log \( \text{ID} \); \( y \) the log OE; \( a \) the intercept with the \( y \)-axis, and \( b \) is the slope.

Infectivity (%) was determined according to the following equation:

\[
\frac{\text{ID}}{\text{total dose}} \times 100
\]

The difference of infectivity to 100 was defined as the inhibitory activity (IA, %).

2.3.1. Statistical analysis

The non-parametric Mann–Whitney U test was used to compare mean oocyst counts between neonatal mice. The level of significance was determined at \( P < 0.05 \).

3. Results

3.1. Efficacy of KENO\(^\text{TM}\)COX in destroying C. parvum oocysts

Observed microscopically, untreated oocysts appeared refracting, spheroid to ellipsoid, 5.0–5.5 μm, containing a residual body and sporozoites (Fig. 1a). After disinfectant contact, as soon as 15 minutes, oocystal content was expelled from some oocysts through the oocyst suture (Fig. 1b and c) leaving empty oocysts in disinfectant medium (Fig. 1d and e). This suture is a structure in the oocyst wall, which span around 1/3 the circumference of the oocysts at one pole which may be dissolved, resulting in a slit-like opening by which the sporozoites exit the oocyst during excystation for example. But contrary to excystation where 4 motile sporozoites actively exit through the oocyst suture, here, the oocyst content (grouped sporozoites) was released (Fig. 1b); sporozoites were motionless. The percentage of empty oocysts increased in time. Numerous empty oocysts and oocystal contents were observed.
suspended in disinfectant medium after 1 h contact time (Fig. 1e) or aggregated (Fig. 1f).

Count results are summarized in Table 1. The initial theoretical number of oocysts was of $10^7$ oocysts/tube i.e. $10^6$ oocysts/ml at CT0, before the contact with KENO™COX. The average of the 6 counts was 987,500 oocysts/ml for C, 1,000,000 and 999,167 oocysts/ml for K2 and K3 respectively; C, K2 and K3 were not significantly different ($P > 0.05$). These averages were not different from the target mean of $10^6$ oocysts/ml.

After 2 h contact at room temperature ($\approx 22^\circ C$) with water (C) or KENO™COX (K2 and K3), the mean number of oocysts in C (900,833 oocysts/ml) was significantly different from that counted at CT0 ($P < 0.05$). The recovery rate was 91.2%. This difference may be explained by the successive washings and centrifugations. The mean numbers of oocysts in K2 (100,833 oocysts/ml) and K3 (81,667 oocysts/ml) were significantly different from those counted at CT0 ($P < 0.05$) and significantly different from C ($P < 0.05$). The recovery rate was 10.1% and 8.2% i.e. an 89.9% and 91.8% reduction in the number of oocysts. When compared to control C recovery rate, KENO™COX recovery rate was 11.07% and 8.99% in K2 and K3, respectively. Expression of KENO™COX efficacy as the oocyst lysis rate derived from the difference of the recovery rate from 100 was 88.93% and 91.01%.

Thus, contact with KENO™COX 2% or 3% for 2 h lysed 88.9% or 91.0% of C. parvum oocysts; however, among all the intact oocysts counted in K2 and K3 some ones were not refracting, they appeared dark and might be not viable or not infectious. Their infectivity was tested by inoculation to neonatal mice.

3.2. Oocyst infectivity test in C57 Bl/6 neonatal mice

Infectivity results of non-lysed C. parvum oocysts after 2 h contact with KENO™COX 2% and 3%, assessed in neonatal mice and compared to C. parvum control oocyst infectivity, are shown in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Oocyst Count</th>
<th>Inoculated Oocysts</th>
<th>Oocyst Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>987,500</td>
<td>5 x 10^5</td>
<td>2,600,700</td>
</tr>
<tr>
<td>K2</td>
<td>1,000,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td>999,167</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In control group C, after inoculation with disinfectant-unexposed oocysts ($5 \times 10^5$ oocysts/mouse), all mice excreted oocysts and the mean oocyst excretion was 2,600,700 oocysts per mouse. Oocyst excretion was...
Assessment of Cryptosporidium parvum

Table 2

<table>
<thead>
<tr>
<th>Disinfectant Code</th>
<th>Contact time (h)</th>
<th>Recovery rate (%)</th>
<th>% disinfectant efficacy (%)</th>
<th>% efficacy corrected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>987,500 ± 16,355</td>
<td>91.2</td>
<td>8.8</td>
</tr>
<tr>
<td>KENOTM COX 2%</td>
<td>0</td>
<td>1,000,000 ± 20,976</td>
<td>10.1</td>
<td>89.9</td>
</tr>
<tr>
<td>KENOTM COX 3%</td>
<td>0</td>
<td>999,167 ± 13,571</td>
<td>8.2</td>
<td>91.8</td>
</tr>
</tbody>
</table>

\(\%\) recovery rate = \([\text{oocyst number at CT2}] / [\text{oocyst number at CT0}]\) × 100.

\(\%\) disinfectant efficacy = 100 – recovery rate.

\(\%\) efficacy corrected.

The results of the oocyst excretion of mice inoculated with serial dilutions of a suspension of unexposed C. parvum oocysts are shown in Fig. 2. Oocysts were counted in intestine content of mice inoculated with 250, 500 and 5000 oocysts, but the excretion was below the threshold of detection when 100 oocysts were inoculated per mouse. The calibration curve showed a clear linear association after logarithmic transformation of values \(R^2 = 0.9748\).

Inoculation with the dose of 500,000 oocysts per neonatal mouse after 2 h incubation in KENOTM COX 2% KENOCOX 2% markedly reduced oocyst excretion and hence infectivity of the C. parvum oocysts administered. Compared to the theoretical line, KENOTM COX 2% reduced the number of 500,000 infectious oocysts to 48.6 ingested oocysts \(\text{Log} = 1.687\) i.e. 0.00972 \% of the control dose. The corresponding inhibitory activity is 99.99 \%. In increasing the concentration of KENOTM COX from 2 \% to 3 \%, infection was completely prevented in neonatal mice. The inhibitory activity of KENOTM COX 3 \% is 100 \%.

4. Discussion

Minimizing exposure of neonates (calf, kid, lamb) to C. parvum oocysts is one of the key factors in controlling cattle cryptosporidiosis. The results of this study show that KENOTM COX, a commercial amine-based bactericidal disinfectant, was able to destroy or inactivate C. parvum oocysts. As reported by Najdrowski et al. (2007) the disinfectant resulted in the weakening of the oocyst wall suture but contrarily to their observations where excystation was completely prevented in neonatal mice. The inhibitory activity of KENOTM COX 3 \% is 100 \%.

Fig. 2. Oocyst production 6 days post inoculation of C57 Bl/6 neonatal mice with 100, 250, 500, and 5000 C. parvum oocysts (logarithmic scales) and regression line allowing the determination of the dose corresponding to the infection level in K2 and K3 groups.

numbers infective oocysts present in inocula administered to the mice. In our study 4 out of 16 mice excreted oocysts. The response logit calculated as the natural logarithm of the quotient of the proportion of animals infected divided by one minus the proportion of animals infected was −0.477 corresponding according to Korich et al. model to a log dose between 1.5 and 1.7 i.e. an infective dose between 33 and 55 oocysts. In our study, the oocyst excretion of mice inoculated with KENO\(^{TM}\)COX 2%-exposed oocysts was reduced by 97.5%. The linear correlation between oocyst excretion of mice infected with serial dilutions of a suspension of untreated oocysts after logarithmic transformation of values, allowed to determine the oocyst dose ingested by mouse inoculated with disinfectant-exposed oocysts at 48.6 oocysts (value included between 33 and 55 oocysts determined according Korich model); then, KENO\(^{TM}\)COX 2% inhibitory activity was 99.99% instead of 59 oocysts determined according Korich model); then, oocysts at 48.6 oocysts (value included between 33 and 55 oocysts determined according Korich model).

The response logit calculated as the natural logarithm of the proportion of mice infected with serial dilutions of oocysts of mice infected with serial dilutions of oocysts was reduced by 97.5%. The linear correlation between oocyst excretion of mice infected with serial dilutions of a suspension of untreated oocysts after logarithmic transformation of values, allowed to determine the oocyst dose ingested by mouse inoculated with disinfectant-exposed oocysts at 48.6 oocysts (value included between 33 and 55 oocysts determined according Korich model); then, KENO\(^{TM}\)COX 2% inhibitory activity was 99.99% instead of 91.0% in vitro. A minimum inhibitory activity of 95% is claimed as the threshold for certification of a product as suitable for disinfection of coccidia (Eimeria) (Daugschies et al., 2002; Shahiduzzaman et al., 2010).

Joachim et al. (2003a) and recently, Shahiduzzaman et al. (2010) reported inactivation of C. parvum with two cresolic disinfectants: Neopredisan\(^{®}\) 135-1 and Aldeccol\(^{®}\) XD, at 1% and 4% concentrations for 2 h of contact and considered that the recommended application of 4% Neopredisan\(^{®}\) for the disinfection of coccidia (Eimeria, Isospora) for 2 h includes inactivation of C. parvum. However, with Eimeria, sporulation inhibition does not have to be the only test to judge the disinfectant efficiency. Some disinfectants (e.g. Neopredisan) showed very effective on C. parvum oocysts and on E. tenella unsporulated oocysts (>95% disinfectant activity) have a minor activity (<60% disinfectant activity) on E. tenella sporulated oocysts (personal observations); because the majority of oocysts in broiler houses are sporulated it is essential for a disinfectant to be effective against Eimeria sporulated oocysts.

The values obtained with KENO\(^{TM}\)COX indisputably exceeded the postulated 95% threshold of inhibitory activity values and, consequently the product may also be certified under these conditions for disinfection of C. parvum oocysts. Efficacy of KENO\(^{TM}\)COX as that of both cresolic disinfectants should be explored in field conditions, especially in the presence of heavy organic loads. Amine compounds are known to be efficient in presence of organic matter. A preliminary test showed oocyst destruction of 82.04% with 4% KENO\(^{TM}\)COX directly in contact with faeces for 2 h contact time. However, for any disinfection program, a complete cleaning should be implemented prior to disinfectant application to obtain the best results. The aim is not to create a pathogen-free environment, but reduce the infection pressure to an acceptable level. Calves infected with only a few oocysts do not show symptoms, develop progressively immunity becoming less susceptible to the parasite towards 3 weeks of age but shedding oocysts in the environment. This is currently observed at the beginning of the calving season. Beyond a threshold, diarrhoea and even mortality appear in newborn calves heavily infected. The infection pressure must be maintained below the critical threshold of diarrhoea appearance by good hygiene, good management practices and using regularly a suitable disinfectant.

To conclude, under the conditions of our study, in vitro disinfection using KENO\(^{TM}\)COX 2% and 3% for 2 h resulted in a significant reduction of viability and infectivity of C. parvum oocysts, as assessed in neonatal mice. The inhibitory activities observed (99.99% and 100%) show that the product KENO\(^{TM}\)COX at 2% and 3% in water is an effective disinfectant and constitutes a means to inactivate C. parvum oocysts which might be used to disinfect rearing material and premises.

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References


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