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Calicophoron daubneyi (Digenea: Paramphistomidae): The efficacy of anthelmintics in naturally infected cattle

Calicophoron daubneyi (Diginea: Paramphiszomidae): Účinnost anthelmintik u přirozeně infikovaného skotu

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ABSTRACT

The effectiveness of anthelmintics in the treatment of paramphistomosis in farm animals in the Czech Republic has not been sufficiently investigated. This study was focused on selected breeds of beef cattle. Identification of *Calicophoron daubneyi* was performed by sedimentation and verified by PCR using the 5.8S rRNA gene. Animals were treated specifically for *C. daubneyi* infection only. Totally 400 positive animals were included in the study. The anthelmintic Closamectin (Closantel/Ivermectin) was evaluated as the most effective veterinary medicinal product with an efficiency of 80%, followed by Distocur (Oxyclozanid) with an efficiency of 78%, Aldifal (Albendazol) with an efficiency of 71% and Levatum Plus (Ivermectin/Clorsulon) with an efficiency of 62%.

Keywords: anthelmintics, *Calicophoron daubneyi*, coprology, parasite, cattle

ABSTRAKT

Účinnost anthelmintik při léčbě paramfistózy u hospodářských zvířat v České republice není dostatečně prozkoumána. Tato studie byla zaměřena na vybraná plemena masného skotu. Identifikace *Calicophoron daubneyi* byla provedena sedimentací a ověřena pomocí PCR s využitím genu 5,8S rRNA. Zvířata byla léčena specificky pouze na infekci *C. daubneyi*. Do studie bylo zařazeno celkem 400 pozitivních zvířat. Jako nejúčinnější veterinární léčivý přípravek bylo vyhodnoceno anthelmintikum Closamectin (Closantel/Ivermectin) s účinností 80 %, následované Distocurem (Oxyclozanid) s účinností 78 %, Aldifalem (Albendazol) s účinností 71 % a Levatum Plus (Ivermectin/Clorsulon) s účinností 62 %.

Klíčová slova: Anthelmintika, *Calicophoron daubneyi*, koprologie, parazit, farmová zvířata

INTRODUCTION

Paramphistomosis is a gastrointestinal parasitic disease caused by digenetic trematodes of the Paramphistomidae family. Rumen flukes infect ruminants and consist of a number of different species of the Paramphistomidae family (Gunathilaka et al., 2018). This family includes, for example, the genus *Paramphistomum*, Calicophoron, Cotylophoron, Ceylonocotyle, Gastrothylax, Fischoederius, Carmyerius, Gastrodiscus and Pseudodiscus (Torres-Acosta et al., 2012; Zhao et al., 2017; Kotze et al., 2020). Rumen fluke parasites impact hugely on livestock productivity by affecting growth rates, fertility, meat quality, wool or milk production, and they sometimes cause mortality. In subtropical and tropical areas, the infection leads to economic losses related to mortality and low productivity (Spence et al., 1992; Spence et al., 1996; Rieu et al., 2007). In general, rumen fluke is only considered to be of clinical relevance when ruminants are exposed to massive burdens of infective stages while grazing. Immature stages that migrate in the duodenum can cause clinical symptoms like weakness, diarrhoea and mortality (Spence et al., 1996; Rieu et al., 2007; Mason et al., 2012).

The developmental cycle of Paramphistomidae is indirect and includes mollusks. The life cycle is very similar to the life cycle of liver fluke *Fasciola hepatica*. Movable ciliated miracidia, which hatch from eggs coming from the faeces of an infected host, next infect amphibians or aquatic snails (Moazeni and Ahmadi, 2016; Hotessa and Kanko, 2020). In this environment, they reproduce asexually and develop into cercariae. These are excreted continuously for up to one year and develop into encysted metacercariae in the vegetation. Encysted metacercariae enter to definitive host by ingestion of infected grass or hay (Moazeni and Ahmadi, 2016; Hotessa and Kanko, 2020). At the juvenile stage, parasites excystation in the small intestine and feed on the intestinal mucosa. As they grow, they migrate upward into the reticulum and rumen, where they live at the adult stage excretion eggs into the environment together with the faeces of the host (Mavenyengwa et al., 2010; González-Warleta et al., 2013).

Paramphistomatidae are the best-known trematodes of the rumen and reticulum in ruminants (Horak, 1971; Rolfe and Boray, 1987). For many years, interest in paramphistomes was limited to the tropics and sub-tropics, as the group was considered relatively non-pathogenic and unimportant in temperate regions (González-Warleta et al., 2013). Currently, sharp increases in rumen fluke infections have been recorded throughout Europe (Sangster et al., 1991; Arias et al., 2011; Malrait et al., 2015; Červená et al., 2022).

Despite their ubiquitous presence, our current knowledge of the fundamental molecular and developmental biology of rumen flukes is limited, particularly in comparison to other trematodes of veterinary significance such as the liver fluke, *Fasciola hepatica*. Immunological methods and detection of serum antibodies have not been sufficiently studied. As a result, the diagnosis of paramphistomosis in live animals still depends on the detection of eggs in the faeces (Spence et al., 1996; Torres-Acosta and Hoste, 2008; Liu et al., 2014). Since the infection caused by trematodes leads to economic losses, it is necessary to apply anthelmintics to the animals. Anthelmintics are drugs of synthetic or biosynthetic origin and their effectiveness is affected by the mechanism of their action (Torres-Acosta et al., 2012). Benzimidazoles are the broadest group of drugs used in trematode therapy. Substances of this chemical group act not only against adult forms, but also against all developmental stages. The mechanism of action first leads to disruption of the formation of the mitotic spindle, without which cell division cannot be completed. After that, also the function of cytoplasmic microtubules is broken. These are responsible for the parasite's food intake. If parasite does not get glucose, its energy reserve (in the form of glycogen) is slowly depleted and it dies. Benzimidazoles have the ability to bind better to microtubules of parasites than hosts. This chemical group includes, for example, oxyclozanide, albendazole, closantel and others (Sangster et al., 1991; Elard et al., 1999; Humbert et al., 2001; Coles and Stafford, 2001; Dorny et al., 2011).

Perhaps due to their lack of recognition and poorly understood pathogenicity, no anthelmintic drugs (asides from a single formulation of oxyclozanide licensed only in France: Douvistome) are currently available with a label claim for rumen fluke control in Europe. Numerous studies, both *in vitro* and *in vivo*, have attempted to verify the efficacy of existing anthelmintics against a range of rumen fluke species (Rolfe and Boray, 1987; Paraud et al., 2009; Arias et al., 2011). Currently, oxyclozanide (normally marketed as a treatment for liver fluke) is the drug of choice to control both immature and mature paramphistome infections, although this drug does not appear to have been tested against immature *C. daubneyi* specifically (Selemetas et al., 2015). Additionally, research into diagnostic tools for paramphistomosis has been very limited (Anuracpreeda et al., 2013; Huson et al. 2017) and currently no diagnostic test has been developed for the identification of prepatent infections Huson et al. 2017).

The aim of this article was (1) to improve awareness of the effectiveness of drugs against *C. daubneyi* in cattle and (2) to compare the effectiveness of the most commonly used anthelmintics based on the results of the faecal egg count reduction test (FECRT) against *C. daubneyi* in cattle.

MATERIALS AND METHODS

All study procedures were approved in accordance with the "Act on the protection of animals used for scientific purposes" of the Czech Republic. This act is in accordance with the EU Directive (No. 2010/63 / EU) on the protection of animals used for scientific purposes and with the decision of the Ministry of Agriculture of the Czech Republic No. 22036/2019-MZE-18134. Permission to collect study samples was granted by participating farms.

The study took place in 2022. Four hundred heifers at the age of 13-16 months, naturally infected and positive for *Calicophoron daubneyi* were included in the study. In all heifers, the presence of *C. daubneyi* in the faeces was verified molecularly already before the start of treatment. Genomic DNA extraction was performed from 300 mg of feces using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and further processed according to the manufacturer's recommendations except for the use of 0.5 mm glass beads. For molecular analysis, primers were modified according to the original methodology of Rinaldi et al. (2005) and Itagaki et al. (2003). The amplification region of the 5.8S gene was used with the primer sequence (5´ – 3´) F: TAGGCAATGTGGTGGTGTT and R: TTGCACGTCAGAATCGCT, annealing temperature 55.2 °C, length 1,156 bp. Before starting the treatment, the feces of all animals in the study were examined molecularly and by sedimentation for the presence *C. daubneyi*. After the start of the treatment, the effectiveness of individual anthelmintics was monitored and animal feces were examined only by sedimentation (Cabaret and Berrag, 2004).

The following breeds of beef cattle were observed in this study: Charolaise, Aberdeen Angus (red and black variants), Hereford and Salers. The cattle sampled in this study had never received anthelmintic treatment before. The pasture on which the individuals were stabled at the time of the study was previously used as a temporary paddock for horses, and thus the transmission of *C. daubneyi* infection from the pasture environment is minimized. During the entire study period, the animals were on pasture with the possibility of a shelter, drinking water and ad libitum access to hay and haylage.

Cattle were divided into four experimental groups of 100 individuals, stratified according to the number of faecal eggs, to ensure that each group included animals with the same range of egg numbers (eggs per gram (EPG) < 50.4) and the same representation of breeds. The control group of animals consisted of 40 untreated individuals that were kept out of contact with the treated animals throughout the study. The intensity of infection in the control group was in the range of EPG 49.1 - 83.7 throughout the study period (summer 2022 EPG 49.1 — 76.2; autumn 2022 EPG 53.1 — 83.7). These animals were not treated with any anthelmintics during the entire study period. There was no movement of animals between these experimental groups during the course of the study, or contact of individuals from other groups.

Each group was given the same anthelmintic with an effect on flukes twice a year (spring/autumn), according to the veterinarian's recommendation. The first group was administered the anthelmintic Aldifal (Albendazol; Mikrochem s.r.o., Slovakia), the second group was administered Closamectin (Ivermectin/Closantel; Norbrook Ltd., Czech Republic), the third group was administered Distocur (Oxyclozanid; Merial SAS, Belgium), and the fourth group of cattle was administered Levatum Plus (Ivermectin/Clorsulon; Zoetis Italia Slr., Roma; Table 1). The assignment of anthelmintics to the experimental groups was completely random. All experimental groups were balanced (breed, EPG). Individual anthelmintic application was performed by a veterinarian. The samples of faeces were collected from the anus of each individual always on the day of treatment and 14 days after anthelmintic application in accordance with the methodology of Nzalawah et al. (2018) and Rinaldi et al. (2005). They were examined by sedimentation for the presence of *C. daubneyi*.

In this study, the species variability of the selected cattle breeds for natural infection was not monitored. The efficacy of veterinary medicinal products has been studied for the benefit of cattle. The study evaluated the overall effectiveness of individual drugs against natural *C. daubneyi* infection in cattle. The effectiveness of the drug depending on the method of application (inject or oral) was not evaluated either, this is a topic for another study, as well as the susceptibility of cattle to reinfection with *C. daubneyi*, which was also not monitored in this study.

Sedimentation

In a 150 ml glass beaker, 4 g of fresh faeces was intensively mixed with distilled water using a spatula. The suspension was filtered through a fine sieve (100 µm). The resulting filtrate was allowed to stand for 20 minutes in order to decant the supernatant. Sedimentation and decantation were repeated 4 times (in the first phase 1/3 of the upper half of the solution was aspirated, in the second and third phase 1/2 of the solution was aspirated and in the fourth phase the solution was removed up to the sediment). The sediment was evaluated microscopically on a slide at a low magnification from 10× to 40× (Thienpont et al., 1986, modified).

FECRT

Individually collected faecal samples were stored in collection tubes at 4 °C during transport and processing (within 24 hours). The efficacy of the veterinary medicinal products was assessed using the faecal egg count reduction test according to the methodology of Kochapakdee et al. (1995) and Mooney et al. (2009). The test determination provided an estimate of anthelmintic efficacy. This was done by comparing the number of eggs in treated and untreated hosts. The principle of the test was to evaluate the reduction/increase of parasites in the faeces before and after the treatment with veterinary medicinal products. The efficacy of the drug was calculated by using a mathematical formula and was further expressed as the percentage of reduction of parasites in the faeces. The methodology was adopted

Table 1. Design of drug application

Drug (active substance)	Application	Dosage (ml/50 kg)
Aldifal (Albendazol)	Oral	3.7
Closamectin (Ivermectin and Closantel)	Injected	2.0
Distocur (Oxyclozanid)	Oral	1.0
Levatum Plus (Ivermectin and Clorsulon)	Injected	1.5

according to the studies of Kochapakdee et al. (1995) and Mejía et al. (2003).

FECRT before and after individual treatment evaluation

E1 is the pre-treatment EPG (day 0), and E2 is the post-treatment EPG (day 14). Each host serves as its own control.

$$FECRT = 100 \times (1 - (E_2/E_1))$$

Arithmetic means EPG (Kochapakdee et al.,1995).

Veterinary medicinal products have been administered as recommended by the manufacturer (Table 1). It was always done separately for each individual drug.

Statistical methods

Analyzes were evaluated based on FECRT results. Each time, two groups were evaluated, namely on the day of drug administration (day 0) and after drug administration (day 14). The effectiveness of veterinary medicinal products was evaluated according to the methodology of Kochapakdee et al. (1995) and Mooney et al. (2009). All analyzes were performed in Statistica 6.0 software (StatSoft ČR, Prague, Czech Republic). Efficacy was determined by calculating the faecal egg count reduction (FECRT) and was considered effective when the calculated FECRT was ≥ 95% and the 95% lower confidence limit (Humbert et al., 2001; Kochapakdee et al., 1995; Keyyu et al., 2008; Sanabria et al., 2013; Keyyu et al., 2006). The percentage reduction in FECRT was calculated for each drug individually.

RESULTS

In the course of this study, a decrease of *C. daubneyi* eggs in the feces was recorded in all animals already after the first application by 43.7% (spring 2022), during the second application (autumn 2022) the number of eggs in the feces and the number of infected animals decreased by only 36.7%. There were no significant differences in the number of eggs in feces (EPG < 50.4) between the experimental treatment groups at day 0 (spring 2022 and autumn 2022). All animals in the group excreted *C. daubneyi* eggs in their feces throughout the study period. However, the frequency of faecal excretion decreased slightly after treatment (EPG < 26.1 there was no significant decrease or increase in infection in the control group, which was not treated with any drugs. At the second anthelmintic application, the intensity of infection was without a significant difference in the number of eggs in the feces (EPG < 50.3). Individuals in whom the presence of *C. daubneyi* in the feces was proven by sedimentation (with ≥ 1 egg/g) were considered positive. During the whole study, no cattle were sold, moved or died. The occurrence of intermediate hosts in the pasture was not recorded during the study. The pasture was not waterlogged in any part in the monitored year. Individuals in the control group were positive for the presence of *C. daubneyi* eggs in their feces throughout the study.

Closamectin (Closantel/Ivermectin) was evaluated as a highly effective anthelmintic against *C. daubneyi* according to the results of FECRT at the first application with an efficiency of 84%, followed by Distocur (Oxyclozanid) with an efficiency of 81%, then Aldifal (Albendazol) 76% and Levatum Plus (Ivermectin/Clorsulon) 75%. After the second application, Closamectin (Closantel/Ivermectin) was 77% effective, followed by Distocur (Oxyclozanid) with 75% effectiveness, then Aldifal (Albendazol; 65%) and Levatum Plus (Ivermectin/Clorsulon; 48%). The resulting values are shown in Table 2. After the second application of anthelmintics, the efficacy decreased slightly, but no statistically significant difference was proven ($P > 0.05$). Further studies to verify the resistance of parasites to anthelmintics will be required.

None of the monitored individuals was treated prophylactically in the past, so the risk of resistance is less likely. Cattle were grazed during the study on pasture primarily used for horses. When applying the drugs, the application dose was strictly observed according to the manufacturer's recommendations (Table 1).

Table 2. Efficacy of tested drugs (%) according to test results of the faecal egg count re-duction test

	The commercial name of the drug					
	Closamectin (Closantel/ Ivermectin)	Distocur (Oxyclozanid)	Aldifal (Albendazol)	Levatum Plus (Ivermectin/ Clorsulon)	Control group	Therapy
Day 0 (n=)	100	100	100	100	40	
EPG	50.2 (49.4–51.0)	50.4 (49.6–51.2)	50.1 (49.3–50.9)	50.2 (49.4–51.1)	50.3 (49.1–51.8)	
Day 14 (n=)	53	46	68	58	40	Spring 2022
EPG	8.1 (3.2–13.0)	9.6 (4.7–14.5)	12.1 (7.2–17.0)	12.4 (7.5–17.3)	68.2 (53.1–83.7)	
FECRT (%)	84 (78.1–89.6)	81 (74.7–87.2)	76 (69.7–82.2)	75 (68.7–81.2)	×	
Day 0 (n=)	100	100	100	100	40	
EPG	50.1 (49.3–50.9)	50.2 (49.4–51.0)	50.3 (49.5–51.1)	50.1 (49.3–50.9)	59.3 (53.1–66.2)	
Day 14 (n=)	65	56	69	63	40	Autumn 2022
EPG	11.7 (7.8–17.6)	12.5 (7.6–17.4)	17.3 (12.4–22.2)	26.1 (21.2–31.0)	68,3 (53.1–83.7)	
FECRT (%)	77 (70.9–83.4)	75 (69.0–81.5)	66 (59.7–72.2)	48 (42.1–54.6)	×	
Average effectiveness (%)	**80**	**78**	**71**	**62**	×	

N = the number of positive animals; "(± 95.0 %)" confidence interval; $P > 0,05$; "×" = not rated.

DISCUSSION

The results of this study (FECRT) clearly show that the effectiveness of veterinary medicinal products ranged from 62 to 80%. Specifically, it was the anthelmintic Closamectin (Closantel/Ivermectin; 80%), Distocur (Oxyclozanid; 78%), Aldifal (Albendazol; 71%) and Levatum Plus (Ivermectin/Clorsulon; 62%). In the Czech Republic, there are no equivalent findings on the inefficacy of benzmidazole treatment against *C. daubneyi*. These are the only preparations registered in the Czech Republic. The effectiveness of drugs can only be compared at the level of foreign sources. The active ingredient oxyclozanide is almost never found in veterinary medicinal products available in the Czech Republic and is mainly applied outside the Czech Republic (Huson et al., 2017; Avramenko et al., 2017).

In older studies, oxyclozanide was found to be highly effective against adult flukes when its dose was increased one and a half times from the standard dose of 1 ml per 50 kg (Spence et al., 1996). Recent studies, however, do not confirm this (Rolfe and Boray, 1987; Doyle and Cotton, 2019). In this study, standard dosage was followed for all applied anthelmintics (Table 1). Arias et al. (2013) found a high anthelmintic effect of oxyclozanide in dairy cattle (99% FECRT) using a standard dose. However, the effectiveness of Closamectin (Closantel/ Ivermectin) in this study does not confirm the results compared to the study by Nzalawah et al. (2018), where the ineffectiveness of closantel was found at the standard dose. Other studies assess the effectiveness of *Fasciola hepatica* and paramphistome mixed infection in cattle (Ico-Gómez et al., 2021).

This fact can be influenced by the environment, resistance, or other factors such as cattle breed or age. It is known that underdosing is a factor that can lead to reduced drug effectiveness (Spence et al., 1996; Rolfe and Boray, 1987). A study by Huson et al. (2017) and Babják et al. (2018) shows that it is not always necessary to choose a treatment. According to them, it is more effective to keep parasites at a relevant level (Huson et al., 2017; Babják et al., 2018). After the second application of anthelmintics, the EPG value was higher (EPG 11.7 - 26.1) than the EPG value after the first application (EPG 8.1 - 12.4). However, the EPG value before the application of the drugs varied from 50.1 to 50.4 the entire time. The EPG value in the herds was unified at the beginning of the study to minimize differences on day 0 (control).

This fact indicates a theoretical reduction in the effectiveness of the selected anthelmintic and a further study is needed, where the resistance of the parasite to the applied anthelmintic will be evaluated. In this study, the effectiveness of anthelmintics was monitored 14 days after drug administration in accordance with the study by Flanagan et al. (2011), who recommends to determine the drug effectiveness by FECRT on the 14th day after the treatment in domestic ruminants infected with flukes, as this sampling time allows complete removal of eggs from the host.

However, the development cycle of flukes is much longer, for excysting the trematode takes more than a month to reach the rumen (Moazeni and Ahmadi, 2016). Yet Feces samples were collected on the 14th day, according to the methodology (Flanagan et al., 2011). Similar findings were observed by Brockwell et al. (2014), who stated that, according to the FECRT results, the effectiveness of the anthelmintic is already demonstrable on the 7th day after the treatment in fluke-infected cattle.

Studies by Cabaret and Berrag (2004) and Holm et al. (2014) confirmed that FECRT results can be affected by various factors such as environmental conditions, level of infection in the herd and anthelmintic effectiveness. The effectiveness of the treatment is further confirmed by studies by Vadlejch et al. (2014) and Martínez-Valladares et al. (2015). The study by Vadlejch et al. (2014), Gunathilaka et al. 2018 and Vineer et al. (2020) point out that biosecurity conditions must be observed on each farm to prevent the spread of infectious diseases outside the farm.Although our study did not show a change in the condition of the animal before and after treatment, the study by Cerda et al. (2019) shows a completely opposite case for fluke infection in a herd.

Identification of the fluke *C. daubneyi* was performed according to Swart (1954), Ferreras et al. (2014), and Jones et al. (2015). Morphological and morphometric features confirming *C. daubneyi* infection were identified by microscopic imaging and DNA was verified by PCR sequencing (Rinaldi et al. 2005; Thienpont et al., 1986; Lotfy et al., 2010). The sequence was identical to studies of this kind (Martínez-Ibeas et al., 2013). *Calicophoron daubneyi* is the most common fluke of the genus Paramphistomatidae in farm animals. This fact is also confirmed by studies by Gordon et al. (2013) and Atcheson et al. (2020).

In this study, only natural *C. daubneyi* infection was monitored in animals. Apart from the study on nematode infection Babják et al. (2018), the effectiveness of medicaments for natural paramphistomosis infection in farm animals was not monitored in the Czech Republic. In New Zealand and South America, there are nematodes infecting cattle that are resistant to multiple anthelmintic groups (active ingredients contained in veterinary medicinal products) (Mejía et al., 2003; Atcheson et al., 2020; Ziółkowska et al., 2012). A better understanding of the extent of resistance is needed in Europe to develop and support more sustainable approaches to parasite control.

However, in this study, only the effectiveness of the most commonly applied anthelmintics against *C. daubneyi* was monitored, resistance was not monitored here. A database of European published and unpublished research on resistance to gastrointestinal helminths (GIN) and liver fluke (*Fasciola hepatica*) was collected by members of the European COST action "COMBAR" (Combating anthelmintic resistance in ruminants) and combined with

data from a previous systematic review of resistance in GIN. A total of 197 publications on GIN resistance were available for analysis. There are 535 studies in 22 European countries covering the period from 1980 to 2020 (Vineer et al., 2020; Jones et al. 2015), but no similar studies have been conducted in Paramphistomatidae. As medications are often used at doses higher than the minimum required to kill most parasites in terrain, this dosage could induce a high frequency of resistance alleles (Doyle and Cotton, 2019; Wolstenholme et al., 2004). Parasite resistance is now widespread across Europe and there are still gaps in our knowledge in some regions and countries. The risk of resistance is a major threat to the sustainability of modern ruminant animal production. It causes reduced productivity and threats to animal health (Vineer et al., 2020; Jones et al. 2015; Atcheson et al., 2020; Ziółkowska et al., 2012). The establishment of resistance is difficult to assess on the sole measure of FECR since it depends strongly on the experimental procedure (with or without controls) and the evaluation of the reduction (Cabaret and Berrag, 2004).

CONCLUSIONS

The incidence of flukes in the Czech Republic has increased significantly in recent years. Waterlogged pastures and the problem with medicines are the main factors. In the Czech Republic, there are no registered medicines with the active substance oxyclozanide for the treatment of flukes, their application in the herd is only possible with an exceptional permit. In the event of a strong infection, it is necessary that every breeder take this measure. The results are useful in designing further studies on the seasonality of infection, intermediate host environments, interactions with other flukes and appropriate control strategies for paramphistomiasis. The results of this study are beneficial to any cattle breeder where natural paramphistomatidae infection is a problem. The dynamics of the occurrence of *C. daubneyi* during the year, and the associated design of the most suitable time for the application of drugs and their frequency, is a subject for further study.

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