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RESEARCH ARTICLE

Comparative mucin production in biliary and intestinal epithelia of opisthorchiasis-susceptible and non-susceptible animal models

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Abstract

Objectives: This study aimed to investigate the mucin production change within biliary and intestinal epithelia following *Opisthorchis viverrini* infection in susceptible and non-susceptible animal models.

Materials and Methods: We examined archived paraffin blocks from a time-series study involving two animal models of opisthorchiasis. Hamsters and Balb/c-R/J mice were categorized as susceptible and non-susceptible models, respectively. Histopathological and histochemical (AB-PAS staining) techniques were used to assess mucin production, specifically the mucin index, in both biliary and intestinal epithelia. Statistical analysis was performed to compare differences between epithelium types in both animal models and between non-infection (NI) and *O. viverrini*-infected (OV) groups.

Results: Goblet cell metaplasia of biliary epithelium was detected in the OV group of both animal models. The response was early in the mice model and late in the hamster model. Histochemical examination revealed mixed-type mucin in hamster bile duct and villus of duodenum, with acid-type mucin in duodenal submucosal glands and neutral-type among zymogen granules. Mice predominantly displayed acid-type mucin in the bile duct and mixed-type mucin in the intestine. The intestinal epithelium consistently exhibits a higher mucin index than the hamster's bile duct. In contrast, no statistical difference exists between mice's biliary and intestinal epithelia after day 2 post-infection.

Conclusions: Goblet cell metaplasia was observed as a response in the biliary epithelium, producing mucin in response to OV infection of both susceptible and non-susceptible models. This response was less pronounced in the intestine.

Keywords: Mucin production, *Opisthorchis viverrini*, bile duct, intestine, host susceptibility

Introduction

Opisthorchiasis caused by the liver fluke (*Opisthorchis viverrini, O. viverrini*), is a significant global public health concern, particularly in countries with extensive wetlands like those in the Greater Mekong Sub-region (Sripa et al., 2018b). This parasite is recognized as the primary agent responsible for the development of a deadly form of bile duct cancer known as cholangiocarcinoma or CCA (IARC, 1994). However, it is essential to note that not all *O. viverrini*-infected patients develop CCA (Mairiang, 2017). Due to the limitation in human study, additional research on opisthorchiasis in animal models is required. Hamsters is a model of choice while mice is not suitable model when determining the infection outcome (Boonmars et al., 2018). Previous studies have reported the successful *O. viverrini* infection and maturation within the biliary system of the Golden Syrian hamster, a susceptible animal model that exhibits histopathological characteristics resembling those observed in *O. viverrini*-infected humans (Bhamarapravati et al., 1978; Sripa, 2003; Boonmars et al., 2009). In contrast, the mouse model is non-susceptible to *O. viverrini* infection, as indicated by the parasite elimination from the host (Boonmars et al., 2009; Tangkawattana et al., 2023; Thongrin et al., 2023).

Research on the non-susceptible murine model exposed to intestinal worms has shown their capacity to expel helminths, often through mechanisms involving goblet cell hyperplasia and increased mucin production at an early stage of infection (Fujino and Fried, 1996; Van Panhuys et al., 2013; Cortes et al., 2015; Hasnain et al., 2017b). Goblet cells are responsible for mucin production, which constitutes the main component of mucus, providing crucial protection to the mucosa against injury. Additionally, the production of mucin and the overexpression of goblet cells are closely associated with specific immune responses aimed at eliminating helminths (Hasnain et al., 2013). Our recent study showed that the biliary epithelium of non-susceptible host mice exhibited higher expression of acid-type mucin, which is resistant to mucinase (OV-M60-like-1), a significant component of *O. viverrini* excretory/secretory product, OVES (Wendo et al., 2022). Suggested mice react strongly to expel the fluke, and the mucin alteration represents a defensive mechanism within the biliary epithelial mucosa compared to hamsters. This response may be linked to the characteristics of how the intestinal mucosa responds to gut-dwelling worms in the mouse model (Ishikawa et al., 1993). The acid-type mucin overproduction in murine models has proven to be an effective defense mechanism against intestinal helminths (Soga et al., 2008; Tsubokawa et al., 2009; Tsubokawa et al., 2015; Hasknain et al., 2017b).

The pathogenic effects of *O. viverrini* infection have been extensively documented (Lvova et al., 2012; Suyapoh et al., 2021; Tangkawattana et al., 2023; Thongrin et al., 2023), particularly in the early stages of infection, leading to liver, common bile duct, gall bladder, and kidney abnormalities (Sripa, 2003; Sripa and Kaewkes, 2002). However, it is worth noting that there is limited evidence regarding changes or abnormalities in the host's intestine in response to the infection. Most research has focused on the pathogenesis in the liver and bile ducts, with comparatively less attention directed toward intestinal responses following *O. viverrini* infection. Since both the duodenal and biliary mucosa are affected in the early stages of *O. viverrini* infection, and distinct susceptibility among experimental animals has been documented, it becomes imperative to conduct a histopathological evaluation of the duodenum and bile duct in both susceptible and non-susceptible hosts. The mucin expression in the biliary and intestinal mucosa of susceptible and non-susceptible animal models following *O. viverrini* infection remains largely unexplored. Such a gap leads to our objective to investigate mucin alteration in the bile duct compared to the intestine (duodenum) of the susceptible and non-susceptible animal models following *O. viverrini* infection.

Materials and Methods

Animal models of infection

Archived paraffin block specimens of 60 male golden Syrian hamsters (*Mesocricetus auratus*) aged six weeks and Balb/c-R/J mice aged six weeks under the

National Research Council (NRC) Project for the fiscal year 2019 were used. Hamsters were taken from the Animal Model Facility Unit, Faculty of Medicine, Khon Kaen University, and the mice were obtained from the Nomura Siam International Company and raised in the Northeast Laboratory Animal Center (NELAC) Khon Kaen University at the Animal Biosafety Level 3 (ABSL-3). The Animal Ethics Record Committee has approved the ethical consideration of animal model usage No. IACUC-KKU-78/61. The paraffin block specimens were divided into 2 groups adapted from the initial experimental design. *O. viverrini* metacercariae preparation and animal infection following the methods described by Sripa and Kaewkes (2002). Accords ing to the previous experiment, each animal in the infected group of mice and hamsters received 50 *O. viverrini* metacercaria taken from naturally infected cyprinoid fishes by intragastric intubation (Sripa and Kaewkes, 2002; Pinlaor et al., 2013). The *O. viverrini*-infected (OV) and non-infected control groups (NI) of hamsters and mice were separated into time points of infection subgroups: 1, 2, 7, 14, and 28 days post-infection (dpi). This study was accepted by the Animal Ethics of Khon Kaen University (IACUC-KKU-57/63).

Histopathological studies

The archived paraffin embedding liver and duodenum were selected from all animals. They were re-cut at 4 µm in thickness and placed on the positively charged microscopic slide (Superfrost Plus®, Shandon, USA). Dee paraffinized and hydrated tissues were stained with a routine histological stain hematoxylin-eosin (H&E) for histopathological identification, as described by (Bancroft et al., 1990). The structural differences of the nucleus were stained with Harris Hematoxylin (Merck®, Germany). At the same time, the cytoplasm and connective tissue were colored contrastingly in pink, red, or orange with eosin (Sigma-Aldrich®, Germany).

Histochemical studies of mucin types in bile duct and intestine

Combining Alcian Blue (AB) pH2.5 with Periodic Acid Schiff staining (ABPAS) determined low acidity of carboxylated simple mucin and neutral mucins. The staining technique was employed with a minor modifications protocol (Bancroft et al., 1990). The positive charge of Alcian Blue detected the negative charge of the simple acid mucin and could not detect the non-charged neutral mucin. Hence, the Periodic Acid Schiff (PAS) reagent has been raised with an adjusted pH 2.5 of Alcian Blue. The PAS recognized the structure of saccharide units upon the 1.2 glycol linkage to produce aldehyde, colored with Schiff's reagent (McManus, 1948). Hydrated tissue was stained with AB pH2.5 diluted in 3% acetate buffer (Merck®, Germany) within 1 hour and washed in 3 dips of 3% buffered acetate and tap water for 5 minutes. Slides were stained with Periodic Acid (Sigma-Aldrich®, Germany) for 15 minutes, followed by Schiff's reagent (Sigma-Aldrich®, Germany) for 1 hour. The nuclei were stained with Mayer's hematoxylin (Sigma-Aldrich®, Germany) for 3 minutes, rinsed into tap water for 5 minutes, dehydrated, cleared, and mounted with Permount (Fisher Scientific®, USA).

Data evaluation and statistical analysis

Each stained slide was evaluated under a light microscope. Qualitative data was shown as the histoa pathological alterations of biliary and intestinal epithelial cells. Histochemical detection of mucin types was shown in these categories: blue, magenta, and purple colors determined acid, neutral, and mixed mucin types, respectively. The outcomes represented a systematic random sampling of the liver section of experimental units. The amount of mucin produced by the goblet cells was captured and examined under the light microscope connected to a digital camera. The color interpretation of histochemical reactivity in the goblet cells was quantified by software (ImageJ 1.52a Wayne Rasband, National Institutes of Health, USA), obe tained as a mucin index. The mucin index is the ratio of stained goblet cells in the duodenal or biliary epithelial cells among the 1000-cell population, then designated in pers centage (Theodoropoulos et al., 2001; Lvova et al., 2012). The non-parametric Mann-Whitney U test was raised whenever the data did not meet the normality test to conclude some features between two independent variables using SPSS statistical software version 26.0 (SPSS Inc., USA). Significance is considered at the p-value<0.05.

Results

Histological changes of biliary and intestinal epithelia after *O. viverrini* infection

The study qualitatively demonstrated the differences in histological features in the biliary and intestinal mucosa of non-infected and *O. viverrini*-infected models (Fig. 1 and 2). Notably, goblet cell or secretory-type epithelial cell metaplasia was evident in the biliary epithelium of both hamsters and mice following *O. viverrini* infection. The differences in mucin expression at both locations were also statistically significant (p-value<0.05) through quantitative analysis of the mucin index in the epithelial cell population. For a more comprehensive assessment of mucin production in each location—biliary and intestinal epithelium—comparisons between normal non-infected (NI) and *O. viverrini*-infected (OV) groups are presented in Table 1.

The mucin index of biliary epithelial cells in the OV group significantly differs from the normal non-infected group in the hamster and mice models. The *O. viverrini* infection increases the number of goblet cells and the amount of mucin in the biliary epithelium. However, the infection appears to have no discernible impact on mucin production in the intestinal epithelium. On the other hand, the mucin index comparison between the biliary and intestinal epithelia in each group of NI and OV hamsters and mice is displayed in Table 2. The mucin production significantly differs between the biliary and intestinal epithelia with and without *O. viverrini* infection in hamsters and mice (p-value=0.000). The intestinal epithelium consistently exhibits more goblet cells and greater mucin content than the biliary epithelium.

Distinctive mucin types production of biliary and intestinal epithelia: infection time

The comparison of the mucin index between the biliary and intestinal epithelia in *O. viverrini*-infected hamsters and mice at various stages of infection was subjected to statistical analysis and is presented in Table 3. The results demonstrate a significant difference in mucin production between OV hamsters' biliary and intestinal epithelium on each day of infection (p-value<0.05). In contrast, this significant difference is observed in mice only on days 1 (p-value=0.000) and 2 (p-value=0.000). Notably, the mucin production in the intestine (duodenum) consistently exceeds that in the bile duct throughout the infection period in hamsters, while in mice, this pattern is observed only during the first two days of infection, as indicated by the mean rank values in Table 3). However, an intriguing observation arises during the later stages of infection (days 7 to 28) in mice, with a notable increase in mucin production in their biliary epithelium. Importantly, this increase becomes statistically insignificant compared to intestinal mucin production (p-value>0.05, Table 3). The higher mean rank of biliary epithelium mucin index in mice is detected at day 14 post-infection.

In hamsters and mice, the intestinal epithelium was observed to prominently produce acid and mixed mucin types, with a lower expression of the neutral mucin-type in both NI and OV groups. However, in the NI group and during the early stages of the OV group (up to day 7 post-infection in hamsters and day 2 post-infection in mice), goblet cells were not observable among biliary epithelial cells. Subsequently, goblet cell metaplasia, containing acid and mixed mucin types, became visible exclusively in the intrahepatic bile duct of both animal models in the OV groups (Fig. 1B and Fig. 1D). Notably, mice's bile ducts appeared to respond more vigorously to *O. viverrini* infection compared to hamsters (Fig. 1D and Table 3), primarily producing acid mucin-type after two days of infection. Furthermore, a statistical analysis revealed that the quantity of goblet cells producing acid, mixed, and neutral mucin types was higher in the OV intestinal epithelium than in the biliary epithelium except for the production of acid mucin-type in mice (Table 4).

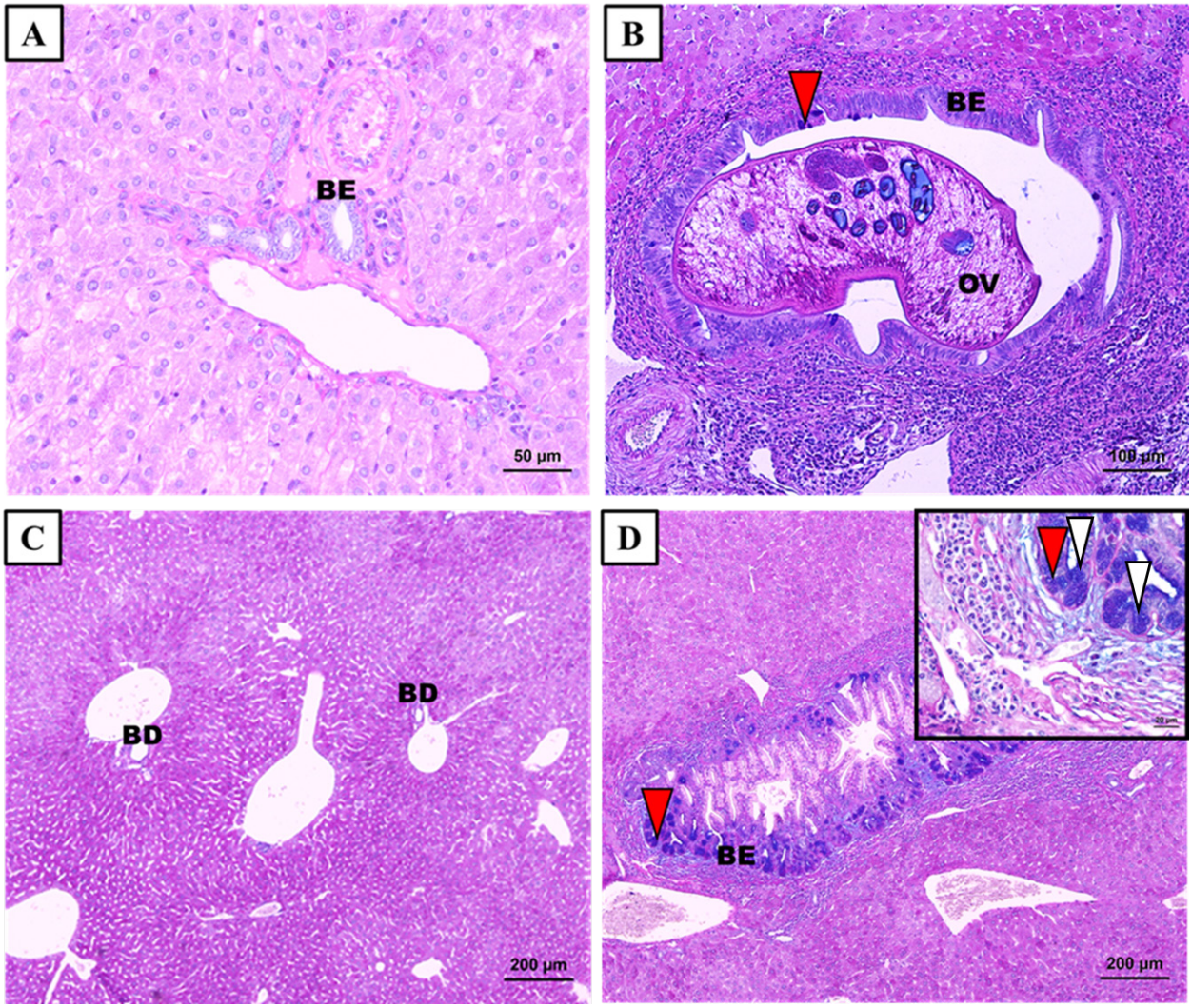

Fig. 1: Comparison of Mucin Production in Biliary Epithelium. Comparative analysis of mucin production in the biliary epithelium was studied in two animal models: hamsters (A, B) and mice (C, D). The expression of mucin in the biliary epithelium was visualized in two groups: the non-infected (NI) group (A and C) and the *O. viverrini*-infected (OV) group 28 dpi (B and D), using Alcian Blue pH2.5-Periodic Acid Schiff (ABPAS) histochemistry. In both hamsters (A) and mice (C) from the NI groups, goblet cells and their corresponding mucin products were conspicuously absent within the biliary epithelial cells (BE) among bile ducts (BD). However, in the hamster OV group (B), goblet cell metaplasia was evident, characterized by the presence of mixed mucin (red arrowhead) within the biliary epithelium (BE). A similar pattern of acid mucin overproduction and goblet cell metaplasia was observed in the mouse OV group (D). Inset: A higher magnification view is provided in the inset, focusing on the overproduction of mixed (red arrowhead) and acid mucin (white arrowhead).

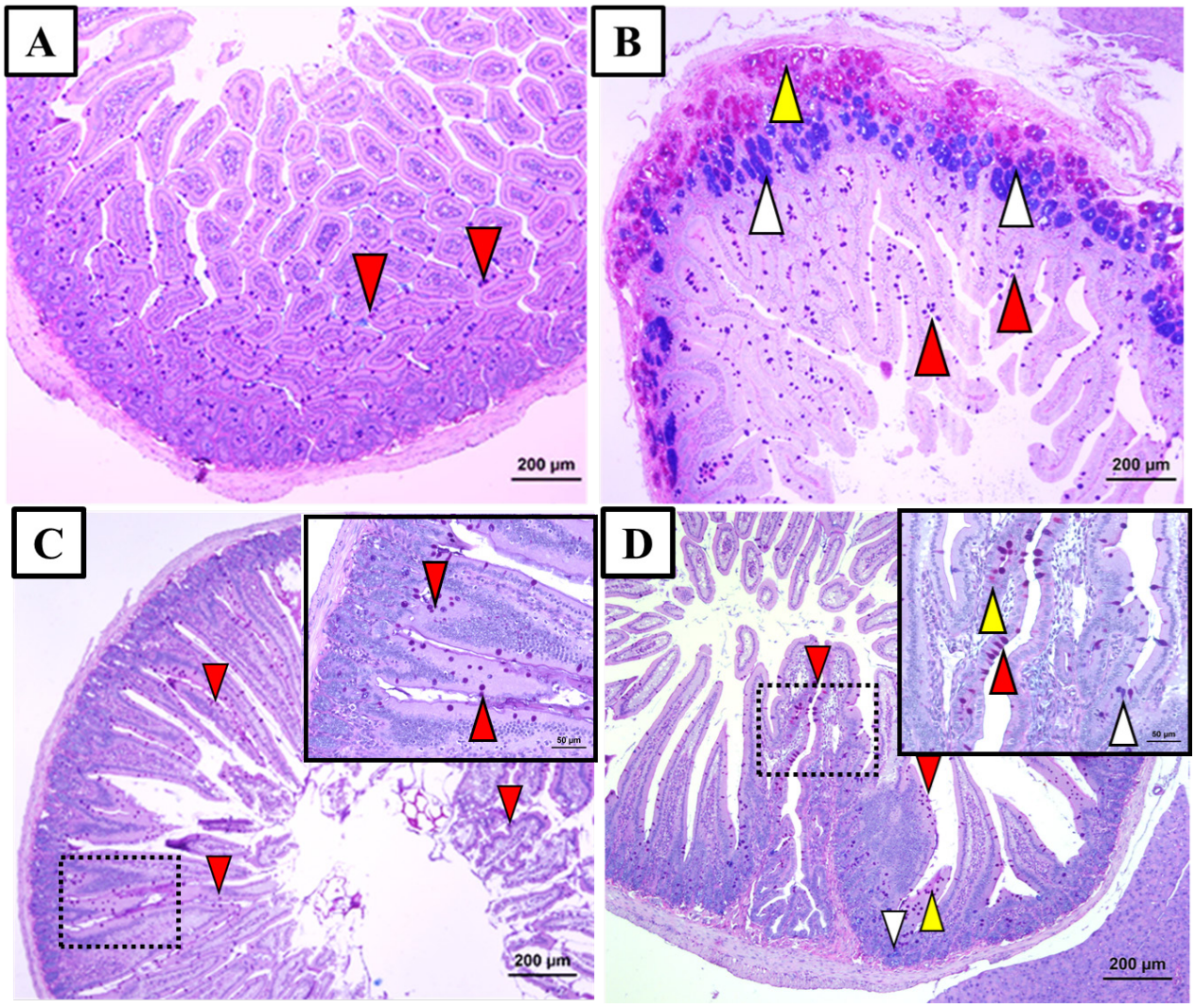

Fig. 2: Comparison of Mucin Production in Duodenal Epithelium. The micrographs show duodenal mucin production in hamster (A, B) and mouse (C, D) models. Alcian Blue pH2.5-Periodic Acid Schiff (ABPAS) histochemistry was used to demonstrate mucin expression patterns in non-infected (NI) (A, C) and *O. viverrini*-infected (OV) 14dpi (B, D) groups, with images captured at 50x magnification. Both NI and OV groups predominantly produced mixed mucin types within goblet cells among villi (red arrowheads) in hamsters and mice. Hamsters displayed acid-type mucin in basal glands with the Paneth cells (white arrowheads) and neutral mucin (yellow arrowheads) among zymogen granules. At the same time mice showed occasional neutral-type mucin in villous goblet cells (yellow arrowheads). Acid-type mucin was also present in basal glands in mice (white arrowhead)—inset: higher magnification (200x) of dashed square area.

Table 1. Statistical analysis of mucin index determination in each location; hamsters and mice's biliary and intestinal epithelia compared the normal non-infected (NI) to *O. viverrini*-infected (OV) group of overall days-post infection.

	Mucin index					
	Hamster			Mice		
Location	Non-infected	OV-infected	p-value	Non-infected	OV-infected	p-value
Biliary epithelium (Mean rank)	85.50	99.50	0.000*	91.00	142.00	0.000*
Median (IQR)	0.00 (0.00-0.00)			0.00 (0.00-0.00)		
Intestinal epithelium (Mean rank)	103.61	89.24	0.072	84.29	76.71	0.301
Median (IQR)	0.17 (0.02-0.27)			0.17 (0.04-0.32)		

IQR=interquartile range
*A P-value from the Mann-Whitney U test less than 005. is considered statistically significant.

Table 2. Statistical analysis of mucin index determination in each group of normal non-infected (NI) and *O. viverrini*-infected (OV) at all infection periods of hamsters and mice in comparison between the biliary and intestinal epithelia.

	Mucin index					
	Hamster			Mice		
Infection status	Biliary epithelium	Intestinal epithelium	p-value	Biliary epithelium	Intestinal epithelium	p-value
Non-infected (Mean rank)	55.50	132.46	0.000*	61.50	152.15	0.000*
Median (IQR)	0.00 (0.00-0.20)			0.00 (0.00-0.09)		
OV-infected (Mean rank)	57.66	129.19	0.000*	81.40	123.30	0.000*
Median (IQR)	0.00 (0.00-0.14)			0.06 (0.00-0.23)		

IQR=interquartile range
*A P-value from the Mann-Whitney U test less than 005. is considered statistically significant.

Discussion

This study provides comparative mucin profiles observed in the biliary and intestinal epithelia following *O. viverrini* infection. However, it is essential to note that our study did not find a statistically significant difference in mucin index between NI and OV intestinal epithelia ($p>0.005$). Suggests that *O. viverrini* infection does not stimulate increased mucin production in the intestine since mucin predominantly covers the gastrointestinal epithelium, serving as a protective component of mucosal immunity. Both secreted and transmembrane mucins form a dynamic defensive barrier capable of responding to enteric infections by adjusting the mucus's production rate, composition, and physical properties (McGuckin et al., 2011). Unlike the biliary epithelium, the intestinal epithelium appears to remain relatively unaffected by the fluke's development, movement, feeding activity with its suckers (which

Table 3. Statistical analysis of mucin production in different periods of *O. viverrini*-infection of the biliary epithelium compared to the intestinal epithelium in hamsters and mice models.

	Mucin index					
	Hamster			Mice		
Days post infection	Biliary epithelium	Intestinal epithelium	p-value	Biliary epithelium	Intestinal epithelium	p-value
1 (Mean rank)	14.00	30.25	0.000*	12.50	32.50	0.000*
Median (IQR)	0.00 (0.00-0.07)			0.00 (0.00-0.04)		
2 (Mean rank)	16.00	37.29	0.000*	15.33	28.25	0.000*
Median (IQR)	0.00 (0.00-0.17)			0.03 (0.00-0.25)		
7 (Mean rank)	12.70	25.75	0.000*	18.58	23.38	0.196
Median (IQR)	0.00 (0.00-0.12)			0.14 (0.00-0.27)		
14 (Mean rank)	8.17	19.25	0.000*	20.54	20.44	0.978
Median (IQR)	0.012(0.00-0.21)			0.14 (0.02-0.25)		
28 (Mean rank)	10.67	17.38	0.032*	16.05	21.56	0.117
Median (IQR)	0.035 (0.01-0.10)			0.12 (0.01-0.23)		

IQR=interquartile range

*A P-value from the Mann-Whitney U test less than 0.05 is considered statistically significant.

Table 4.Statistical analysis comparison of each mucin-type index in all post-infection periods of OV-infected biliary and intestinal epithelium of both animal models

	Mucin index					
	Hamster			Mice		
Mucin types	Biliary epithelium	Intestinal epithelium	p-value	Biliary epithelium	Intestinal epithelium	p-value
Acid (mean rank)	12.17	34.83	0.000*	26.48	22.85	0.379
Median (IQR)	0.04 (0.00-0.17)			0.06 (0.02-0.16)		
Mix (mean rank)	12.00	35.50	0.000*	15.83	38.30	0.000*
Median (IQR)	0.03 (0.00-0.14)			0.15 (0.00-0.24)		
Neutral (mean rank)	21.00	26.88	0.011*	18.00	35.15	0.000*
Median (IQR)	0.01 (0.00-0.00)			0.00 (0.00-0.01)		

IQR=interquartile range

*A P-value from the Mann-Whitney U test less than 0.05 is considered statistically significant.

can cause mechanical injury), or the metabolic products of the liver fluke (Sripa et al., 2018a).

In response to the infection, biliary epithelial cells could transform into goblet cells, while normal non-infected biliary epithelial cells remained unaltered with no positive reaction to ABPAS staining. It has been well-documented that such goblet cell metaplasia lesions result from *O. viverrini* infection (Sripa, 2003; Sripa et al., 2018b; Suyapoh et al., 2021). The goblet cell phenotype is not detectable in the non-infected biliary epithelium because the cholangiocyte activation typically requires injury to undergo further cellular senescence and a senescence-associated secretory phenotype (SASP) during the progression of infection (Cheung et al., 2018). That is, throughout the infection periods in this experiment, the mean rank of the mucin index in the biliary epithelium was consistently lower than in the intestinal epithelium. This outcome may also be associated with cholangiocytes making up only about 5% of the liver's cell population (Banales et al., 2019). Another reason that increased mucin production in the biliary epithelial mucosa could be a secondary compensatory response to cholestasis (Zhu et al., 2016). The mucin index between the biliary and intestinal epithelia of *O. viverrini*-infected hamsters exhibited statistically significant differences at days 1 to 28 of infection, showing a meager amount of biliary epithelial mucin during this time. In mice, these differences were observed only on days 1 and 2. Considerably, it indicated that mice expressed higher levels of mucin in their biliary epithelium as much as in the intestine after day 2 of infection. It can be explained by day 7 of infection, the size of the worm within the bile duct had increased to three times that of the juvenile stage and continued to increase, reaching six to ten times by weeks 2 and 4 of infection (Nithikathkul et al., 2007). The development of *O. viverrini* may lead to obstruction and typically triggers initial pathophysiological responses in the biliary epithelium, such as hyperplasia (Sato et al., 2018). The smaller size of the bile duct in mice could contribute to their significant reactions in increased mucin production, especially during the initial phase due to irritation (Glaser et al., 2009). Suggests that biliary epithelial cells play an active role in hepatocyte repair, which is closely linked to the mucosal immune system of the intestine (Banales et al., 2019). This reaction may also involve cell transformation and proliferation in response to the growth of *O. viverrini* and the secretion of antigenic molecules by the parasite, acting through an immunological mechanism (Adams, 1996). The proinflammatory secretions may initiate crosstalk between activated cholangiocytes and T cells, leading to inflammatory responses, proliferation, and fibrosis in the biliary epithelium (Pinto et al., 2018; Strazzabosco et al., 2018).

Given that, there is specificity in glycoconjugate interactions at the host-parasite interface during the metacercariae and adult stages (Talabnin et al., 2013), the determination of mucin types in different niches of each stage may indeed play a crucial role in the host-parasite interaction. A high level of acid mucin production is observed in the biliary epithelium of mice infected with *O. viverrini*, and this observation was statistically insignificant when compared to the mucin index of the intestinal epithelium. Previous studies have suggested that changes in the terminal chain glycosylation of mucins can play a vital role in either allowing or rejecting helminths in the gut (Ishikawa et al., 1993; Ishikawa, 1994; Maruyama et al., 2002; Yamauchi et al., 2006). The overproduction of acid mucin alters mucus permeability and viscosity, facilitating the trapping of the worm, inhibiting parasite motility, and feeding capacity. These alterations can lead to parasite clearance, which is believed to be regulated by Th2-type immune responses (Webb et al., 2007; Soga et al., 2008; Hasnain et al., 2017a).

Conclusions

In both susceptible and non-susceptible animal models, significant changes in mucin production were observed in the bile duct after being infected with *O. viverrini*. However, similar changes were not evident in the intestinal epithelium. Notably, mucin enhancement in the bile duct of mice became noticeable after day 2 post-infection and eventually reached a level similar to that seen in both the normal non-infected and *O. viverrini*-infected

intestinal mucin indexes. Additionally, acid mucin production was pronounced in mice's O. viverrini-infected biliary epithelium. These findings suggest that mucin types' glycan configuration and composition are likely species-specific and tissue-specific, which may contribute to different responses to *O. viverrini* infection. However, further investigation is needed to understand the mucin glycoconjugate glycan's role in host susceptibility to *O. viverrini* infection comprehensively. Findings could shed light on the mechanisms underlying varying host responses to the parasite and provide insights into potential targets for intervention and control strategies.

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Conflict of interest statement

The authors declare that we have no conflict of interest in this work.

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