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ORIGINAL ARTICLE

Detection of salivary antibodies to crude antigens of *Opisthorchis viverrini* in opisthorchiasis and cholangiocarcinoma patients

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Abstract *Opisthorchis viverrini* (*O. viverrini*; known as human liver fluke) is a major health problem in the northeastern region of Thailand. Infection with *O. viverrini* is the cause of hepatobiliary disease and cholangiocarcinoma (CCA). Previous studies demonstrated specific antibodies to crude *O. viverrini* antigens in serum from *O. viverrini*-infected patients. However, no studies have measured specific antibodies to *O. viverrini* antigens in saliva from patients with opisthorchiasis and CCA. The objective of the study was to detect specific antibodies to crude *O. viverrini* antigens in saliva from patients with opisthorchiasis and CCA, and to evaluate their use for diagnosis of *O. viverrini* infection. Saliva samples from 23 control subjects, 30 opisthorchiasis patients, and 38 CCA patients were collected. ELISA was established for detection of salivary IgA and IgG to crude *O. viverrini* antigens. ANOVA was used to compare salivary IgA and IgG levels among groups. Salivary IgA to crude *O. viverrini* antigens in CCA patients was significantly higher than controls (p= 0.007). Salivary IgG in CCA patients was significantly higher than opisthorchiasis patients and controls (p=0.010 and p<0.001, respectively). The cut-off value from salivary IgG test demonstrated higher accuracy for positivity of *O. viverrini* infection than salivary IgA. In conclusion, specific antibodies to crude *O. viverrini* antigens were detected in saliva of patients with opisthorchiasis and CCA. Salivary antibodies reflect serum immune response to *O. viverrini* infection, and salivary IgG tends to be a good candidate for diagnosis of *O. viverrini* infection.

Keywords Cholangiocarcinoma · ELISA · Opisthorchiasis · *Opisthorchis viverrini* · Saliva

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Introduction

Opisthorchis viverrini (*O. viverrini*) is one of the fish-borne trematodes, known as liver fluke. *O. viverrini* infection or opisthorchiasis is a major public health problem in the Mekong River Basin, especially in Thailand, Lao People's Democratic Republic (Lao PDR), Cambodia, and Vietnam [1–3]. In Thailand and Lao PDR, there is *O. viverrini* infection in approximately 10 million people [4] and 67 million are at risk of infection [5]. *O. viverrini* has a complicated life cycle with two intermediate hosts including freshwater snail and fish [6]. In area where sanitation practices are poor and sewerage infrastructure is inadequate, people with *O. viverrini* infection pass their stool containing these parasite eggs into natural water reservoirs. *O. viverrini* eggs are eaten by *Bithynia* snails. In the snail, the parasites undergo several developmental stages and are released from the snail as free-swimming cercariae which

then encyst in Cyprinid fish as metacercariae. Humans act as the definitive host for the parasite by eating raw or uncooked Cyprinid fish containing infective metacercariae. Metacercaria excyst in human duodenum and ascend via the ampulla of Vater into the biliary ducts where they become matured *O. viverrini* [6, 7].

O. viverrini mainly inhabits in bile ducts, gallbladder, and pancreatic duct, and pathological consequence of the opisthorchiasis is due to the mechanical irritation and some toxic substances produced from the worms [8]. The histopathologic features of opisthorchiasis include inflammatory reactions, epithelial desquamation, adenomatous hyperplasia, globlet cell metaplasia, periductal fibrosis, and granuloma formation. The severity of opisthorchiasis is associated with both intensity and duration of *O. viverrini* infection [9]. Previous studies, both in human and animal models, demonstrated the carcinogenic potential of *O. viverrini* to cause cholangiocarcinoma (CCA) [10–13]. In addition, one study reported that elevated anti-*O. viverrini* antibody titers increased the risk for development of CCA by up to 27-fold [14]. It was hypothesized that chronic inflammation due to *O. viverrini* infection might cause DNA damage and finally followed by oncogenic mutation. This pathological condition in the bile duct possibly transforms the injured cholangiocytes into malignant cells [13]. A number of recent molecular studies demonstrated several potential target molecules for diagnosis [15, 16]. However, a supply of well-defined antigens of *O. viverrini* is still lacking and a difficulty in cultivation of the parasite in vitro [15].

Although previous studies reported specific antibodies to *O. viverrini* antigens in serum from *O. viverrini*-infected patients [17–20], informative data on specific antibodies to *O. viverrini* antigens in other secretory fluid are limited. No studies have investigated specific antibodies to crude *O. viverrini* antigens in saliva from patients with opisthorchiasis and CCA. Salivary antibodies originate mainly from salivary glands with some contribution from serum antibodies via the gingival crevicular fluid. Thus, salivary antibodies can reflect both secretory and systemic immune responses. In addition, the collection of saliva samples is easy and non-invasive, when compared with blood samples. Therefore, the aim of the present study was to detect specific salivary antibodies to crude *O. viverrini* antigens, and to evaluate their potential use for diagnosis of *O. viverrini* infection.

Materials and methods

Participants and collection of human saliva

During October 2006 to April 2008, 91 participants including 38 CCA patients, 30 opisthorchiasis patients, and 23 control subjects living in Khon Kaen province were recruited in the study. All participants signed consent forms, with the approval of the ethical committee for the use of human subjects for research, Khon Kaen University (HE 480316). Clinical information of participants in each group is as follows. CCA patients consisted of 12 women and 26 men with a mean age of 57±10 years, ranging from 36 to 82 years. CCA patients were diagnosed by clinical appearance and confirmed by histopathologic examination. Regarding the medical records, CCA patients had the history of eating raw or uncooked freshwater fish and taking anti-parasitic medications. Opisthorchiasis patients consisted of 12 women and 18 men with a mean age of 48 ±9 years, ranging from 32 to 61 years. Opisthorchiasis patients were diagnosed by the presence of opisthorchid eggs in fecal samples. The control group consisted of 15 healthy women and eight healthy men with a mean age of 27±8 years, ranging from 22 to 47 years. No opisthorchid eggs were detected in fecal samples of control subjects. All participants refrained from eating or drinking 1 h before saliva collection. Each participant was asked to expectorate whole saliva into a 50-mL centrifuge tube, and a final saliva volume of 3 to 5 mL was collected. All saliva samples were immediately placed on ice for transport. Samples were centrifuged at 4,000 rpm for 10 min. Supernatants were aliquoted and stored at −80°C until the assays were performed.

Preparation of crude *O. viverrini* antigens

Golden hamsters were orally infected by the metacercariae of *O. viverrini*, derived from cyprinid fish. After 8 weeks of infection, these infected hamsters were sacrificed, and matured *O. viverrini* were collected from the livers of the hamsters [21, 22]. Crude *O. viverrini* antigens were prepared by homogenization of matured *O. viverrini* in the presence of 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM L-1-tosylamine-2-phenylethyl chloromethyl ketone, and trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane. The homogenized *O. viverrini* were undergone cell lysis by ultrasonic disintegrator for 30 min, kept at 4°C overnight, and followed by centrifugation at 13,000 rpm for 30 min. Supernatants were aliquoted and stored at −80°C until the assays were performed.

Assays for measurement of specific antibodies to crude *O. viverrini* antigens

We employed ELISA protocol previously described for measurement of IgA and IgG to crude *O. viverrini* antigens in human serum [20]. In brief, 100 μl of crude *O. viverrini* antigens (5 μg/ml) in coating buffer, carbonate-bicarbonate buffer pH 9.6, was added to each well. Microtiter plates were kept at 4°C overnight, and then washed three times

with 0.05% Tween 20 in normal saline (NSST). Two hundred microliters of 3% bovine serum albumin in phosphate buffer saline (PBS) pH 7.2 was added to each well for 1 h at 37°C for blocking nonspecific binding, followed by washing three times with NSST. The wells were incubated with 100 μl of diluted saliva sample (1:2) in incubation buffer, PBS with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), for 1 h at 37°C, followed by washing three times with NSST.

For the determination of salivary IgA to crude *O. viverrini* antigens, 100 μl of biotin-conjugated goat anti-human IgA antibody (1:2,000, Zymed Laboratories Inc., San Francisco, CA, USA) in incubation buffer with 2% skimmed milk was added to each well, followed by incubation for 1 h at 37°C. After washing three times with NSST, 100 μl of streptavidin conjugated peroxidase (1:3,000, Zymed Laboratories Inc.) in incubation buffer was added to each well, followed by incubation for 1 h at 37°C. To estimate salivary IgG to crude *O. viverrini* antigens, 100 μl of horse radish peroxidase-conjugated rabbit anti-human IgG antibody (1:7,500, Zymed Laboratories Inc.) in incubation buffer with 2% skimmed milk was added to each well, followed by incubation for 1 h at 37°C.

After washing three times with NSST, 100 μl of freshly prepared substrate solution containing one *O*-phenylenediamine dihydrochloride tablet (Sigma Chemical Co.), 14 μl of 30% H_2O_2, and 20 ml of 0.05 M citrate buffer (pH 5.0) was added to each well. Incubation was approximately 20 min to observe for the development of color in the wells. The reaction was stopped by adding 100 μl/well of 2 N H_2SO_4 and the absorbances were measure on a microtiter plate reader (TECAN, Sunrise microtiter plate reader, Austria) at 492 nm. All saliva samples in the procedures of ELISA were done in triplicate and the data were calculated from the mean optical density (OD) values of three tests for each sample. Triplicate determinations of each sample were estimated for coefficient of variation. Substrate and H_2O_2 was used as a blank control, and incubation buffer in place of saliva sample was used as a negative control. An inter-plate saliva sample from the control subject group was used to compare plate to plate absorbance variation and thereby calibrating plate to plate discrepancy, accordingly. The sensitivity and specificity of the established ELISA were evaluated using subjects with known status of *O. viverrini* infection. A fecal sample from each subject was examined for the presence of *O. viverrini* eggs, using modified formalin-ether concentration method as a gold standard. In addition, the balance between sensitivity and specificity derived from known positive (n=20) and negative (n=20) cases of opisthorchiasis was chosen to establish the cut-off OD values. The cut-off values were selected to yield the highest accuracy (minimal false negative and false positive results). The OD values of samples which were greater than the cut-off values were considered for positivity of *O. viverrini* infection by salivary antibody test.

Statistical analysis

All data of salivary IgA and IgG to crude *O. viverrini* antigens were analyzed as mean OD values. ANOVA was used to compare the ELISA results of salivary IgA and IgG levels to crude *O. viverrini* antigens among groups. Correlations between salivary IgA and IgG to crude *O. viverrini* antigens in each group were determined by calculating Pearson's correlation coefficient. Significance was established at a p value<0.05.

Results

Measurements of specific salivary antibodies to crude *O. viverrini* antigens

The balance between sensitivity and specificity derived from known positive and negative cases of opisthorchiasis was chosen to establish the cut-off OD values, representing the threshold absorbance for positivity of *O. viverrini* infection. The cut-off OD value is 0.59 by salivary IgA test (Fig. 1a). In CCA patients, the OD values from the ELISA of salivary IgA to crude *O. viverrini* antigens ranged between 0.89 and 4.00; the mean was 2.90 and the standard deviation (SD) was 0.76. The OD values from opisthorchiasis patients ranged between 0.15 and 4.00; the mean was 2.63 and SD was 1.00. The OD values from control subjects ranged between 0.16 and 3.71; the mean was 2.18 and SD was 0.87. The mean OD values of salivary IgA to crude *O. viverrini* antigens in CCA patients were significantly higher than those in controls (p=0.007; Fig. 2). Evaluation for the accuracy of the cut-off OD value for positivity of *O. viverrini* infection by salivary IgA test in opisthorchiasis patients demonstrated that 29 cases (97%) were true positive, and one case (3%) was false negative. In control group, one case (4%) was true negative, and 22 cases (96%) were false positive.

For salivary IgG to crude *O. viverrini* antigens, the cut-off OD value is 0.42 by salivary IgG test (Fig. 1b). The OD values from CCA patients ranged between 0.19 and 3.62; the mean was 1.08 and SD was 0.75. The OD values from opisthorchiasis patients ranged between 0.30 and 1.37; the mean was 0.70 and SD was 0.23. The OD values from control subjects ranged between 0.06 and 0.90; the mean was 0.39 and SD was 0.22. The mean OD values of salivary IgG to crude *O. viverrini* antigens from CCA patients were significantly greater than those in opisthorchiasis patients and controls (p=0.010 and p<0.001, respec-

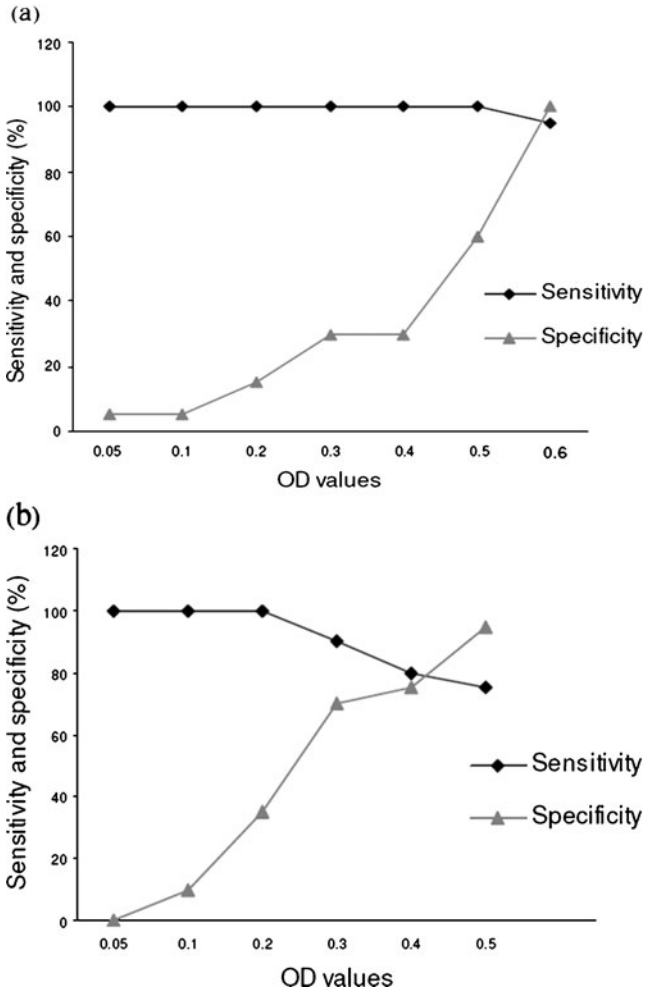

Fig. 1 The cut-off OD value for the positivity of *O. viverrini* infection was established from the intercept between sensitivity and specificity lines. The cut-off OD values for salivary IgA and IgG to crude *O. viverrini* antigens are 0.59 (**a**) and 0.42 (**b**), respectively

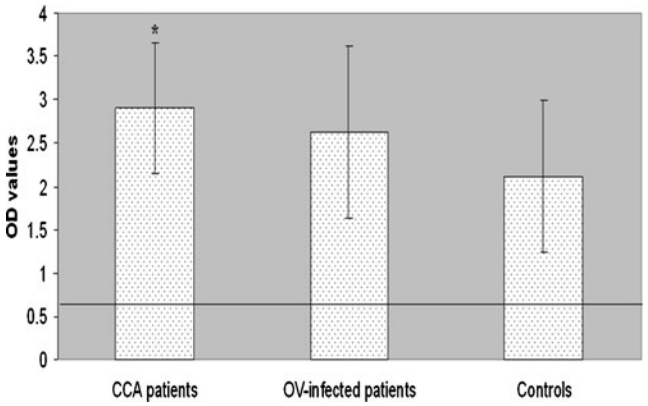

Fig. 2 Mean OD values of IgA antibody to crude *O. viverrini* antigens in saliva from cholangiocarcinoma (*CCA*) patients (n=38), *O. viverrini* (*OV*)-infected patients (n=30), and control subjects (n=23). The *asterisk* indicates a significantly higher level of IgA antibody to crude *O. viverrini* antigens in CCA patients than in control subjects (p=0.007). The *horizontal line* represents the cut-off OD value (0.59) for the positivity of *O. viverrini* infection

tively; Fig. 3). Evaluation for the accuracy of the cut-off OD value for positivity of *O. viverrini* infection by salivary IgG test in opisthorchiasis patients demonstrated that 27 cases (90%) were true positive, and three cases (10%) were false negative. In control group, 15 cases (65%) were true negative, and eight cases (35%) were false positive.

Correlations between specific salivary antibodies to crude *O. viverrini* antigens

Our results demonstrated no significant correlations between salivary IgA and IgG to crude *O. viverrini* antigens in saliva of CCA patients (Fig. 4a). There were no significant correlations between salivary IgA and IgG to crude *O. viverrini* antigens in saliva of opisthorchiasis patients (Fig. 4b). In contrast to the other two groups, significant correlations between salivary IgA and IgG to crude *O. viverrini* antigens in saliva of control subjects were observed (p<0.001; Fig. 4c).

Discussion

To our knowledge, this is the first investigation of specific salivary antibodies to crude *O. viverrini* antigens in opisthorchiasis patients and CCA patients. According to our study, there were three interesting observations. First, our results demonstrated the detection of specific IgA and IgG to crude *O. viverrini* antigens in saliva. Mechanism of inducing salivary antibody to *O. viverrini* remains poorly understood. According to life cycle of this parasite, *O. viverrini* infection occurred by eating uncooked fish

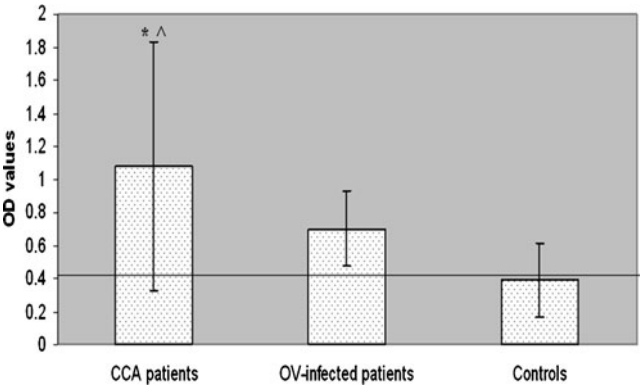

Fig. 3 Mean OD values of IgG antibody to crude *O. viverrini* antigens in saliva from cholangiocarcinoma (*CCA*) patients (n=38), *O. viverrini* (*OV*)-infected patients (n=30), and control subjects (n=23). The *asterisk* indicates a significantly higher level of IgG antibody to crude *O. viverrini* antigens in CCA patients than in OV-infected patients (p=0.010). The *circumflex accent* indicates a significantly higher level of IgG antibody to crude *O. viverrini* antigens in CCA patients than in control subjects (p<0.001). The horizontal line represents the cut-off OD value (0.42) for the positivity of *O. viverrini* infection

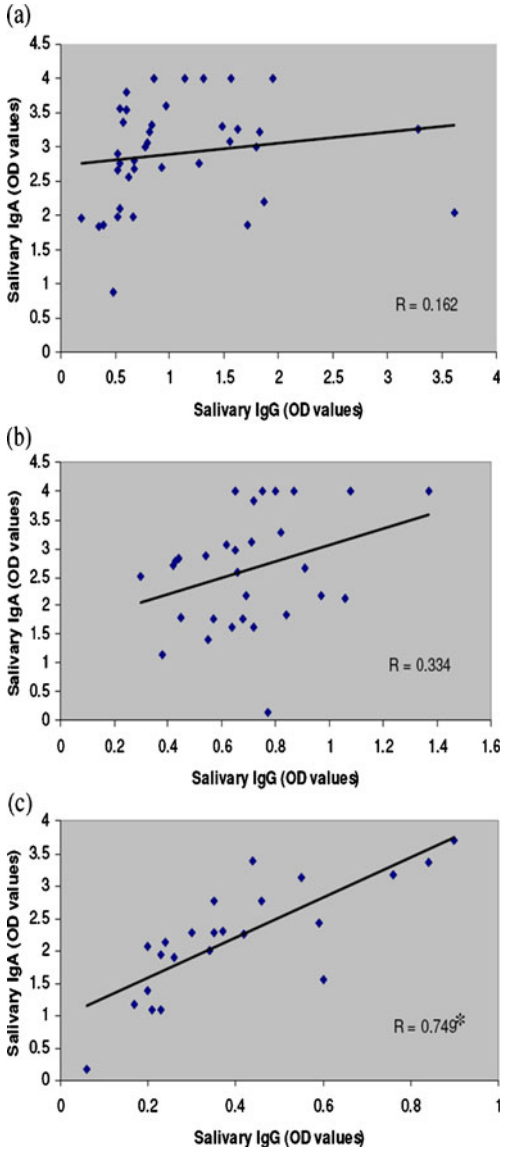

Fig. 4 Correlations between salivary IgA and IgG antibody to crude *O. viverrini* antigens in saliva from cholangiocarcinoma patients (**a**); *O. viverrini*-infected patients (**b**); and control subjects (**c**). The *asterisk* indicates statistically significant correlations between IgA and IgG antibody to crude *O. viverrini* antigens in saliva from control subjects ($p<0.001$)

containing viable metacercarial cyst (infective stage). After ingestion, the metacercaria excyst in the duodenum and rapidly enter the biliary tract via ampulla of Vater for further development. The transit time of the juvenile liver fluke in the gastrointestinal (GI) tract is relative short, approximately 30 min. Thus, the possibility that the parasite directly induces mucosal immune responses of the GI tract and oral compartment is unlikely. By contrast, the adult worms survived in the biliary tract for a long time (up to 5–10 year). There was evidence that the parasite antigens were detected in host tissues such as biliary tract [23]. Therefore, the induction of salivary antibodies to *O. viverrini* may originate from diffusion of the parasite antigens into host tissues probably via bile duct epithelial damage and inflammation, leading to the activation of immune responses in the GI tract and subsequently in the oral compartment. However, further investigations are needed to prove this hypothesis. Previous studies of *O. viverrini* infection were demonstrated elevated levels of serum IgA and IgG to crude *O. viverrini* antigens [17–20]. In agreement with the previous reports, our results demonstrated the increased levels of IgA and IgG to crude *O. viverrini* antigens in saliva of opisthorchiasis patients and CCA patients. One study reported higher serum titers of anti-*O. viverrini* antibodies in CCA patients when compared with opisthorchiasis patients [24]. Similarly, our results demonstrated the higher level of salivary IgG to crude *O. viverrini* antigens in patients with CCA than opisthorchiasis patients. According to the present data, one could speculate that salivary antibodies to crude *O. viverrini* antigens would reflect serum antibody levels, rather than the secretory immune response, to *O. viverrini* infection. However, we could not exclude the possibility that an increase in salivary IgG concentrations might be due to confounding factors such as a mucosal break in the oral cavity or an increase in exudation of the gingival crevicular fluid into whole saliva due to periodontal diseases. Thus, evaluation of the association between salivary and serum IgG levels by assessment of albumin concentrations in saliva would be appropriate to ascertain the source of *O. viverrini*-specific IgG in saliva.

Second, the specificity of salivary antibody test obtained from the present study was lower than serum antibody test [20]. We found the limitation of using the cut-off OD values in salivary IgA and IgG tests for diagnosis of *O. viverrini* infection. In the control group, some individuals demonstrated the positivity of *O. viverrini* infection by salivary ELISA test. There are several possible explanations. For example, an individual who was previously infected with *O. viverrini* and subsequently developed antibodies specific to this parasite may retain level of these antibodies, although this person was treated with anti-parasitic medications, and *O. viverrini* eggs were not detected by fecal examination. In addition, other studies using ELISA techniques for the detection of *O. viverrini* infection revealed the problem with crude cross-reactive antigens [25–27]. It could be speculated that there might be some cross-reaction between salivary antibodies and other parasite antigens. Further investigation to identify specific *O. viverrini* antigens and detection of specific salivary antibodies to these particular antigens would be of importance for future clinical application of saliva for the diagnostic test of *O. viverrini* infection. According to our observations, the cut-off value for positivity of *O. viverrini* infection by salivary IgG test demonstrated higher accuracy

than the cut-off value by salivary IgA test. Thus, if the diagnostic test for *O. viverrini* infection could be performed on saliva samples, specific salivary IgG to crude *O. viverrini* antigens tends to be a good candidate when compared with salivary IgA. However, further clinical investigations of salivary antibody test for *O. viverrini* infection are needed to confirm the potential use.

Finally, our study demonstrated an altered pattern of correlations between salivary IgA and IgG to crude *O. viverrini* antigens in opisthorchiasis patients and CCA patients, when compared with control subjects. The strong correlations between salivary IgA and IgG to crude *O. viverrini* antigens were observed in control subjects, whereas lack of correlations between salivary IgA and IgG to crude *O. viverrini* antigens was demonstrated in opisthorchiasis patients and CCA patients. It remains unknown how co-expression between salivary IgA and IgG to crude *O. viverrini* antigens is regulated under pathological conditions of *O. viverrini* infection and CCA. One study demonstrated differences in profiles of antibody responses to *O. viverrini* infection between bile and serum of opisthorchiasis patients [25]. Thus, it would be of interest to investigate whether there are any differences in profiles of antibody responses to *O. viverrini* infection between saliva and serum derived from opisthorchiasis and CCA patients.

In conclusion, the present study provides evidence that saliva of patients with opisthorchiasis and CCA contains specific antibodies to crude *O. viverrini* antigens. Salivary antibodies reflect serum immune response to *O. viverrini* infection, and salivary IgG tends to be a good candidate for diagnosis of *O. viverrini* infection. Thus, the integration of saliva research into the research area of *O. viverrini* infection, and the intensive studies of salivary antibody test are essential to confirm their potential use for diagnosis of *O. viverrini* infection. In addition, further studies on the association between *O. viverrini*-induced impairment of immunosurveillance mechanisms and development of CCA would be of importance.

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Conflict of interest The authors declare that they have no conflict of interest.

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