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Potential Correlation between Carboxylic Acid Metabolites in *Biomphalaria alexandrina* Snails after Exposure to *Schistosoma mansoni* Infection

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Abstract: Carboxylic acids play an important role in both aerobic and anaerobic metabolic pathways of both the snail and the parasite. Monitoring the effects of infection by schistosome on *Biomphalaria alexandrina* carboxylic acids metabolic profiles represents a promising additional source of information about the state of metabolic system. We separated and quantified pyruvic, fumaric, malic, oxalic, and acetic acids using ion-suppression reversed-phase high performance liquid chromatography (HPLC) to detect correlations between these acids in both hemolymph and digestive gland gonad complex (DGG's) samples in a total of 300 *B. alexandrina* snails (150 infected and 150 controls) at different stages of infection. The results showed that the majority of metabolite pairs did not show significant correlations. However, some high correlations were found between the studied acids within the control group but not in other groups. More striking was the existence of reversed correlations between the same acids at different stages of infection. Some possible explanations of the underlying mechanisms were discussed. Ultimately, however, further data are required for resolving the responsible regulatory events. These findings highlight the potential of metabolomics as a novel approach for fundamental investigations of host-pathogen interactions as well as disease surveillance and control.

Key words: *Schistosoma mansoni*, *Biomphalaria alexandrina*, carboxylic acid, chromatography

INTRODUCTION

Researchers started to explore new array-based technologies to map metabolites to provide signature characteristics for each disease state by taking snap shot of metabolism. Metabolome analysis is the systematic analysis of metabolites present in a cell which represent hundreds of diverse classes of small organic molecules, including amino acids, nucleotides, carbohydrates, carboxylic acids, vitamins, and coenzymes [1].

Metabolomics can be used for 2 major different purposes: screening for differences between metabolic fingerprints of cohorts of populations or to understand the regulatory structure of metabolic pathways and its dynamics [2]. Although the general metabolic needs of parasites for their maintenance, growth, and reproduction are similar to those of all living forms, the specific details of pathways vary greatly [3].

Parasitic helminths have an absolute dependency on carbohydrates for their energy source, and one of the key features of carbohydrate catabolism in parasites is the excretion of a wide range of end products from carbohydrate breakdown; which are di- and tricarboxylic acids, most of which have low molecular absorptivity and thus are poorly detectable compounds by photometric detection [1]. Because of the large number and low concentration of many metabolites, metabolome studies require sensitive, selective, and high throughput separation techniques [4]. There are 2 different approaches to metabolite analysis, i.e., comprehensive and selective [5].

The major physiologic and metabolic mechanisms of snail-schistosome relationships that are crucial for survival, growth, and efficient transmission of parasites, remain partially known [6]. Recent studies on the topic have used high performance thin layer chromatography (HPTLC) to examine the pathobiochemical effects of *Schistosoma mansoni* infection on the amino acid [7], neutral lipid [8], carbohydrate [9], and content of

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Biomphalaria glabrata. Qualitative and quantitative changes in certain analytes were observed as a function of schistosome infection in the snail in all of the afore-mentioned studies.

Carboxylic acids, such as pyruvic, fumaric, malic, oxalic, and acetic play important roles in both aerobic and anaerobic metabolic pathways, as they are involved in the intermediary metabolism of the snail [10]. In a recent work by the authors [11], the potentiality of these acids to be used as diagnostic and therapeutic biomarkers was studied. They were found to be good discriminators between infected and uninfected snails as well as between the different stages of infection. The most suitable acids to be used as drug targets were also detected.

The present study aimed at qualitatively and quantitatively determining these acids in the hemolymph (blood) and digestive gland-gonad complex (DGG) of *B. alexandrina* after infection with *S. mansoni* in order to establish correlations between carbohydrate metabolites in different stages of infection.

MATERIALS AND METHODS

Biological materials were obtained from the Medical Malacology Laboratory, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. A total of 300 snails of an Egyptian strain, including 150 infected cases and 150 controls, were used in this study.

Snail maintenance

Snails were maintained in plastic trays, each containing 10 snails and 1 L of aerated tap water (26 ± 2℃), replaced twice a week, and fed boiled fresh lettuce and blue green algae [12]. Aquaria were cleaned weekly for removal of feces and dead snails [13]. Laboratory reared *B. alexandrina* reaching 6-7 mm in shell diameter was exposed to *S. mansoni* miracidial infection according to Massa et al. [14]. Harvesting of the snails was done as follow: group 1 (G1) included 50 snails of 2 weeks after infection; group 2 (G2) was 50 snails at the time of patency development; and group 3 (G3) was 50 snails of 2 weeks after patency. Uninfected cohort snails of the same shell diameter were maintained in the same manner and harvested at corresponding intervals.

Sample preparation

Hemolymph samples were collected from each snail, after cleaning with a paper towel, using a Pasteur pipette inserted through a tiny hole made in the pericardial region. The samples were then centrifuged at 120 *g* for 5 min to separate the supernatant from hemocytes and cell debris, and then the supernatant was kept at -20℃ till the time of extraction [14].

The DGG tissue extract samples were prepared by dissection free from the snail body, and then carboxylic acid extraction was done using 50% Ringer's solution (Carolina Biological Supply, North Carolina, USA). Centrifugation of the extract was done at 250 *g* for 15 min to collect the supernatants which were stored at -20℃ until use [15].

HPLC analysis

Synthetic standards of acetic, fumaric, oxalic, malic, and pyruvic acids (Sigma-Aldrich, St. Louis, Missouri, USA) in the highest purity grade available were determined by the C18 ion-suppression reversed-phase HPLC according to Lian et al. [16]. Adjusted HPLC graded water as the mobile phase, isocratic elution at a flow rate of 1 ml/min, and the ultraviolet diode array detector at 210 nm with a sensitivity of 0.02 absorbance units were used to obtain chromatograms of the carboxylic acid standards and to determine the retention time of each acid. Calibration curves of each standard were done separately by plotting the peak area of each standard against its concentration, and then pooling of studied carboxylic acid standards was done to obtain calibration curves of studied acid mixtures.

Identification of carboxylic acids in studied samples was made by matching the peak area retention time between standards and studied sample chromatograms. Validation was made by comparing the standards and sample ultraviolet spectra collected by the detector during the separation. Quantitative analyses of the identified acids were done by generating graphs that relate the standard concentrations of the acids to their peak areas. Results were achieved by intrapolation within the calibration curve.

Pooling each of 10 snails was used throughout the study, and the mean of 10 trails for each HPLC analysis was done for each sample. The conversions to part per million concentrations (ppm) were done according to Fishel and Mossler [17]. The concentration (ppm) of each acid in snail DGGs was calculated by multiplying the sample solution concentration interpolated from calibration curve (I) (ppm) by the original sample volume (V) (ml) and division of the product by the mass of the snail DGG (M) (g) and for hemolymph. Carboxylic acid concentration (μg/dl) was calculated by multiplying I times V times 100, and division of the product by the hemolymph volume (HV) (ml) according to Massa et al. [14]. Statistical analysis of the results was done with Microsoft Excel

software using the Pearson correlation analysis.

RESULTS

Descriptive statistics of the studied acid concentrations are shown in Tables 1 and 2, and the correlations between the studied acids in hemolymph (H) and tissue samples (T) in different groups (control group, 2-week post exposure group, shedding group, and 2-week post shedding group) are shown in Tables 3-7.

Control group

In hemolymph samples, a significant positive correlation was found between oxalic and fumaric acids, and a highly significant positive correlation was found between acetic and pyruvic acids. In tissue samples, a significant positive correlation was found between oxalic and pyruvic acids and between acetic and pyruvic acids, a highly significant positive correlation was found between malic and pyruvic acids, and a significant negative correlation was found between fumaric and oxalic acids. Other significant negative correlations were also detected between the concentrations of malic acid in hemolymph and acetic acid in tissue (r=-0.532) and between the concentrations

Table 1. Concentrations (mean±SD) of 5 acids in the 4 groups of hemolymph samples

	Oxalic acid hemolymph	Malic acid hemolymph	Acetic acid hemolymph	Pyruvic acid hemolymph	Fumaric acid hemolymph
Control	335.62±229.87	10.15±6.38	0.04±0.05	0.13±0.22	0.051±0.12
G1	17.92±7.02	0.92±0.49	0.003±0.001	0.014±0.005	0.007±0.011
G2	61.47±48.87	5.99±2.52	0.019±0.004	0.09±0.058	0.0002±0.0001
G3	214.3±103.24	33.85±8.65	0.090±0.062	0.39±0.19	0.013±0.005

Table 2. Concentrations (mean±SD) of 5 acids in the 4 groups of tissue samples

	Oxalic acid in tissue	Malic aicd in tissue	Acentic acid in tissue	Pyruvic acid in tissue	Fumaric acid in tissue
Control	208.71±190.65	13.96±7.06	0.02±0.03	2.16±1.52	0.04±0.02
G1	57.26±24.14	2.63±1.79	0.03±0.01	0.32±0.33	0.003±0.006
G2	232.37±123.69	17.08±6.07	0.17±0.12	0.72±0.63	0.005±0.004
G3	566.92±180.87	17.516±11.89	0.29±0.13	0.71±0.18	0.06±0.01

Table 3. Correlations between the studied acids in hemolymph (H) and tissue samples (T) in control group

		Oxalic H	Oxalic T	Malic H	Malic T	Acetic H	Acetic T	Pyruvic H	Pyruvic T	Fumaric H
Oxalic T	R	0.637								
	P	0.005[b]								
Malic H	R	0.27	0.056							
	P	0.165	0.421							
Malic T	R	-0.016	0.031	-0.168						
	P	0.477	0.457	0.274						
Acetic H	R	-0.041	-0.03	0.096	0.175					
	P	0.442	0.132	0.367	0.267					
Acetic T	R	0.22	0.189	-0.532	0.325	-0.186				
	P	0.215	0.25	0.021[a]	0.119	0.253				
Pyruvic H	R	-.0178	-0.181	0.385	0.118	0.617	-0.315			
	P	0.262	0.26	0.078	0.338	0.007[b]	0.127			
Pyruvic T	R	-0.011	0.451	-0.389	0.655	-0.11	0.572	-0.105		
	P	0.484	0.046[a]	0.076	0.004[b]	0.348	0.013[a]	0.355		
Fumaric H	R	0.462	0.192	0.198	-0.101	-0.065	-0.118	-0.117	-0.256	
	P	0.042[a]	0.247	0.239	0.361	0.409	0.338	0.339	0.179	
Fumaric T	R	-0.003	-0.4	-0.444	-0.236	0.271	0.24	-0.359	-0.194	0.179
	P	0.495	0.05[a]	0.049[a]	0.198	0.164	0.195	0.094	0.244	0.261

R, correlation coefficient; *P*, *P*-value.
[a]Significant; [b]Highly significant.

Table 4. Correlations between the studied acids in hemolymph (H) and tissue samples (T) in the 2 weeks post exposure group

		Oxalic H	Oxalic T	Malic H	Malic T	Acetic H	Acetic T	Pyruvic H	Pyruvic T	Fumaric H
Oxalic T	R	0.41								
	P	0.247								
Malic H	R	0.874	0.092							
	P	0.026[a]	0.441							
Malic T	R	0.057	0.466	-0.222						
	P	0.464	0.215	0.36						
Acetic H	R	0.041	0.885	O.187	0					
	P	0.252	0.023[a]	0.382	0.5					
Acetic T	R	0.578	0.371	0.156	0.494	0.147				
	P	0.154	0.269	0.401	0.199	0.407				
Pyruvic H	R	0.394	-0.613	0.597	-0.678	-0.358	0.037			
	P	0.256	0.136	0.144	0.104	0.277	0.477			
Pyruvic T	R	0.099	0.394	-0.183	0.992	-0.078	0.558	-0.587		
	P	0.437	0.256	0.384	0[b]	0.45	0.164	0.149		
Fumaric H	R	-0.7	-0.564	-0.275	-0.312	-0.46	-0.934	-0.048	-0.352	
	P	0.094	0.161	0.327	0.304	0.218	0.01[a]	0.47	0.281	
Fumaric T	R	-0.657	-0. 543	-0.232	-0.219	-0.488	-0.903	-0.074	-0.256	0.992
	P	0.114	0.172	0.353	0.362	0.202	0.018[a]	0.453	0.339	0[b]

R, correlation coefficient; *P*, *P*-value.
[a]Significant; [b]Highly significant.

Table 5. Correlations between the studied acids in hemolymph (H) and tissue samples (T) in the shedding group

		Oxalic H	Oxalic T	Malic H	Malic T	Acetic H	Acetic T	Pyruvic H	Pyruvic T	Fumaric H
Oxalic T	R	0.006								
	P	0.496								
Malic H	R	0.286	0.758							
	P	0.321	0.069							
Malic T	R	0.004	-0.626	-0.133						
	P	0.498	0.129	0.416						
Acetic H	R	0.776	0.08	0.643	0.262					
	P	0.061	0.449	0.121	0.335					
Acetic T	R	0.925	0.16	0.209	-0.36	0.568				
	P	0.012[a]	0.399	0.368	0.276	0.159				
Pyruvic H	R	-0.702	0.603	0.233	-0.602	-0.491	-0.474			
	P	0.093	0.141	0.353	0.141	0.201	0.21			
Pyruvic T	R	0.94	0.271	0.434	-0.304	0.733	0.966	-0.421		
	P	0.009[b]	0.33	0.232	0.309	0.079	0.004[b]	0.24		
Fumaric H	R	0.884	0.255	0.244	-0.237	0.478	0.915	-0.532	0.874	
	P	0.023[a]	0.34	0.347	0.351	0.208	0.015[a]	0.178	0.026[a]	
Fumaric T	R	0.171	0.175	0.217	-0.581	0.277	0.349	0.34	0.402	-0.028
	P	0.392	0.389	0.363	0.152	0.326	0.282	0.288	0.251	0.482

R, correlation coefficient; *P*, *P*-value.
[a]Significant; [b]Highly significant.

of malic acid in hemolymph and fumaric acid in tissue (r= -0.444) (Table 3).

G1 (2 weeks post exposure group)

In hemolymph samples, a significant positive correlation was found between oxalic and malic acids. In tissue samples, a highly significant positive correlation was found between malic and pyruvic acids, and a significant negative correlation was found between acetic and fumaric acids. Other significant correlations were also detected between the followings: a significant positive correlation between the concentrations of acetic acid in hemolymph and oxalic acid in tissue (r= 0.885) and a

Table 6. Correlations between the studied acids in hemolymph (H) and tissue samples (T) in the 2 weeks post shedding group

		Oxalic H	Oxalic T	Malic H	Malic T	Acetic H	Acetic T	Pyruvic H	Pyruvic T	Fumaric H
Oxalic T	R	0.725								
	P	0.083								
Malic H	R	0.909	0.762							
	P	0.016[a]	0.067							
Malic T	R	0.211	0.681	0.537						
	P	0.367	0.103	0.175						
Acetic H	R	-0.081	-0.025	-0.046	-0.082					
	P	0.448	0.494	0.47	0.448					
Acetic T	R	0.195	0.793	0.223	0.632	-0.062				
	P	0.377	0.055	0.359	0.127	0.46				
Pyruvic H	R	0.438	-0.021	0.352	-0.142	-0.802	-0.336			
	P	0.23	0.487	0.281	0.41	0.051	0.29			
Pyruvic T	R	0.083	-0.478	-0.251	-0.941	0.229	-0.615	0.154		
	P	0.447	0.208	0.342	0.009[b]	0.355	0.135	0.409		
Fumaric H	R	0.333	-0.399	0.193	-0.615	0.073	-0.845	0.5	0.755	
	P	0.292	0.253	0.378	0.135	0.454	0.036[a]	0.196	0.07	
Fumaric T	R	0.695	0.881	0.688	0.589	-0.492	0.707	0.377	-0.475	-0.349
	P	0.096	0.024[a]	0.1	0.148	0.2	0.091	0.266	0.209	0.283

R, correlation coefficient; *P*, *P*-value.
[a]Significant; [b]Highly significant.

Table 7. Correlation between hemolymph and tissue concentrations of the studied acids in the 4 groups

	Oxalic acid		Mlic acid		Aetic acid		Pruvic acid		Fumaric acid	
	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*
Control	0.637	0.005[a]	-0.17	0.274	-0.19	0.253	-0.11	0.355	0.179	0.261
G1	0.41	0.247	-0.22	0.36	0.147	0.407	-0.059	0.149	0.992	0.001[a]
G2	0.006	0.496	-0.13	0.416	0.568	0.159	-0.42	0.24	-0.03	0.482
G3	0.725	0.083	0.537	0.175	-0.06	0.46	0.154	0.403	-0.35	0.283

R, correlation coefficient; *P*, *P*-value.
[a]Highly significant.

significant negative correlation between the concentrations of fumaric acid in hemolymph and acetic acid in tissue (r=-0.934) (Table 4).

G2 (the shedding group)

In hemolymph samples, a significant positive correlation was found between oxalic and fumaric acids. In tissue samples, a highly significant positive correlation was found between acetic and pyruvic acids. Other significant correlations were also detected between the followings: a significant positive correlation between the concentrations of oxalic acid in hemolymph and acetic acid in tissue (r=0.925), between the concentrations of fumaric acid in hemolymph and acetic acid in tissue (r=0.915), and between the concentrations of fumaric acid in hemolymph and pyruvic acid in tissue (r=0.874). A highly significant positive correlation was found between the concentrations of oxalic acid in hemolymph and pyruvic acid in tissue (r=0.94) (Table 5).

G3 (2 weeks post-shedding group)

In hemolymph samples, a significant positive correlation was found between oxalic and malic acids. In tissue samples, a significant positive correlation was found between fumaric and oxalic acids, and a highly significant negative correlation was found between malic and pyruvic acids. Other significant negative correlations were also detected between the concentrations of fumaric acid in hemolymph and acetic acid in tissue (r=-0.845) (Table 6).

Finally, highly significant positive correlations were detected between oxalic acid concentration in both hemolymph and tissue samples in the control group and between fumaric acid concentration in both hemolymph and tissue samples in 2

weeks post-exposure group (Table 7).

DISCUSSION

Despite the difficulties in assessing the causal origin of a specific correlation in metabolomics data, the observed pattern provides information about the metabolic system that allows greater understanding of the underlying process. The observed correlations are properties of the whole system, not of any particular metabolite, enzyme, or reaction [18]. When measuring a population of biological replicates, intrinsic fluctuations may arise due to at least 2 different mechanisms; first, organisms are never actually identical even under identical experimental conditions, there are always inevitable small differences in enzyme concentrations [19], and second, the effects of environmental factors on cellular metabolism [18].

Pooling of each 10 snails was used throughout the study and the mean of 10 trails for each HPLC analysis was done for each sample. Van Saun [20] encouraged the use of pooled samples and stated that, though some variation may be masked, pooled samples may provide an economic alternative to traditional metabolic profiling, as most of the important measures of metabolic status showed minimal differences between pooled and individual samples and found them to be statistically equivalent. He stated that the real challenge of using pooled samples is interpretation. Empirically one can interpret pooled samples by determining how far they deviate from the midpoint of the reference range for the control [19].

Comparing correlations obtained with each single state is more relevant than combining samples of different states, this is particularly important when, in one state there is a high correlation, but not in the other, or when the correlations are both high but of opposite sign [19]. In the present study, some correlations were found to be high within the control group but not in other groups; between oxalic acid concentrations in hemolymph and tissue samples, oxalic and pyruvic acids in tissue samples, pyruvic and acetic acids in hemolymph samples, malic acid concentration in hemolymph, and both acetic and fumaric acid concentrations in tissue samples.

The more striking finding was the existence of reversed correlations, i.e., a situation in which the correlation between 2 metabolites changes its sign [21]. In the present work, although no significant correlation was found between malic and pyruvic acids in all groups in hemolymph samples, a highly significant positive correlation was found between malic and pyruvic acids in the control group and group 1, reversed to a highly significant negative correlation in group 3 in tissue samples. Also, the significant negative correlation that was found between fumaric and oxalic acids in tissue samples in the control group was reversed to a significant positive correlation in group 3. Moreover, the significant negative correlation found between the concentrations of fumaric acid in hemolymph and acetic acid in tissue samples in group 1 was found to be reversed to a positive correlation in group 2, which was then reversed again to a negative correlation in group 3. This points to a marked change in the underlying regulation of the system and possibly reflects the existence of multiple steady states. Indeed, the phenomenon of reversed correlations is also observed in the numerical models of cellular metabolism, involving multi-stationary and switching between different states [22]. However, other causes of reversed correlations are also conceivable and a more detailed evaluation is still needed.

The transition to a different state may not only involve changes in the average levels of the measured metabolites, but also involve changes in their pair-wise correlations. Likewise, a metabolite which shows no significant change in the average level between 2 different experimental conditions may still show an alteration of its pair-wise correlations with other metabolites. This observation leads to an interpretation of the resulting pattern of correlation as a global fingerprint of the state [18].

In our study, the majority of metabolite pairs did not show significant correlations. This was explained by different reports [18,19,21], which stated that metabolite correlations do not necessarily correspond to proximity in the biochemical network as it is noted that neighboring metabolites and directly interacting metabolites in the metabolic network may have little or no correlation. It is not because they are not related, but because the variance in the enzymes that control them also affects them in equal amounts and different directions. This is what happens to most of the metabolite pairs and is the consequence of the systemic nature of metabolic control.

Strong correlations between 2 metabolites are likely explained by the chemical equilibrium between them. Also an interesting prediction, still to be confirmed, is that metabolites sharing conserved moieties should have high correlations and at least one of them being negatively correlated with the others. However, most high correlations may be due to either stronger mutual control by a single enzyme or variation of a single enzyme level much above others. In both cases, it is impossible to identify the responsible enzyme from these data alone, thou-

gh hints can be obtained from the set of metabolites forming correlation clique [22]. Ultimately, further data are required for resolving the responsible regulatory events with protein profiles being the most promising for this effect [18,19].

When 2 metabolites are moderately correlated, it may be due to a large concentration response coefficient towards a common enzyme, or an enzyme that has carries unusually high variance. However, to identify this enzyme requires further data [23].

Metabolites in chemical equilibrium will have nearly perfect positive correlation. As a consequence, metabolites with negative correlation are not in equilibrium. In this case, the correlation does not originate from the enzyme that catalyzes the equilibrium reaction, as the metabolites have very small response towards it [19].

That is, differences or distance in terms of observed correlations should reflect and correspond to differences or distance in states, tissue types, and experimental conditions. The observed correlations should be robust with respect to minor changes in the underlying system, while at the same time they should be susceptible for marked changes in the underlying biochemical system. While the preliminary studies seem to support this view [21,22], large scale comparisons of metabolic correlations are still sparsely reported. Thus, to distinguish the specific mechanisms responsible for an observed correlation does require additional knowledge, and a concluding evaluation of the validity and applicability of large-scale metabolomic correlation analysis requires further experimental verification [18,19].

Consistent differences in both the hemolymph and tissue carbohydrate metabolite correlation profiles of the studied groups represent a promising additional source of information about the state of a metabolic system. However, their interpretation in terms of the underlying biochemical pathways is not straight forward and largely defies an intuitive analysis. These findings highlight the potential of metabolomics as a novel approach for fundamental investigations of host-pathogen interactions as well as for disease surveillance and control.

Identifying enzymes involved in specific metabolic pathway detected only in *S. mansoni*-infected snails may allow the use of certain enzyme inhibitors as target chemotherapeutic control agents. Alteration in the metabolism or metabolic status may also be used as a control measure without killing snails to keep balanced ecology.

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