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CONTENT

Manufacturing performance system for SMEs: A prioritization of KPIs with fuzzy analytic hierarchy process

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ABSTRACT

In today's increasing competitive global market, large and successful manufacturing enterprises have implemented the system of key performance indicators (KPIs) which drives the performance toward the business objectives; however, this is not the case for small-medium sized enterprises (SMEs) which have been increasingly important for any national economy, especially in manufacturing sector. Although the KPIs can ideally be constructed in accordance with the concept of SMART (Specific, Measureable, Attainable, Realistic, Time-related) or balanced scorecard, but SMEs that are lack of limited resources and expertise could rarely afford to build such systems with the appropriate definition and measurement of KPIs. Therefore, the paper aimed to provide systematically the system of KPIs adaptable to SMEs, to prioritize the importance of each proposed KPI with the application of a fuzzy analytic hierarchy process (FAHP), and to instruct the comprehensive deployment of the SMEs' manufacturing performance system.

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1. Introduction

In the global context, SMEs have played a key role of tremendous contribution into national economy, development, and political stability. Specifically, SMEs accounted for over 95% of firms and 60% to 70% of employment in OECD (Organisation for Economic Co-operation and Development) economies (Sergei, 2018), whereas the corresponding numbers in Vietnam were about 98% of total enterprises, 63% of employment, 45% of GDP as reported by USAID (2019). The report also emphasizes the quantity did not match with the quality as around 70% exports were dominated by FDI (Foreign Direct Investment) firms and lead firms also co-located with their foreign suppliers without the involvement of local SMEs. This can be explained by the fact that SMEs have not progressed further on the road of developing their supply chain in the age of globalization (Håkon et al., 2004). One of roadblocks on the way of SMEs to develop their supply chain is productivity issues in which the measurement and improvement of manufacturing activities have still remained the main research area (Sergei, 2018). Furthermore, low performance is waste in different forms in terms of energy, raw-materials, downtime, operations, maintenance, and quality (Carl-Fredrik et al., 2015).

As a well-known principle in industries, *what cannot be measured cannot be improved*, which is also represented by the "check" step in the PDCA (plan-do-check-action) methodology used to measure the success of the business (Bruno & John, 2011). The performance measurement systems are widely utilized by large enterprises, but such systems are not well implemented by SMEs as it should be (Piotr, 2017). One of them is the balanced scorecard that has been introduced for the alignment of business strategies with department objectives; however, the method was proven as an ineffective method for the SMEs due to the prominent barriers to strategic performance (Hudson et al., 2001). One of the barriers is accounted for limitation in understanding of how to measure and manage a performance system as well as potential advantages of implementing such performance systems (Garengo et al., 2004), which was also emphasized by a study of KPIs implemented by SMEs in Vietnam (Ta, 2018). Another research pointed out that a lack of resources and expertise is one of the roadblocks for the deployment of such systems (Pham & Bui, 2014).

To overcome the inherent barriers SMEs have been faced, the paper firstly presents the manufacturing performance system that contains a package of simplified KPIs adapted for SMEs based on the literature review, and then prioritizes them to suit with each SME's context by applying the mathematical model of fuzzy analytic hierarchy process, and finally provides implementation guidelines of such system.

2. Materials and Methods

2.1. Development of KPIs

The proper selection of indicators will sharpen performance and expose areas that need attention. *What gets measured gets done* and *if you can't measure it, you can't manage it* are two of the well-known principles (Bernard, 2012). However, numerous enterprises are working with the improper measures, many of them are incorrectly categorized as KPIs. Due to misunderstanding on performance measures, those enterprises have improperly mixed different indicators. Understanding KPIs plays very critical roles in the success of the business as they function like navigation instruments to understand whether the business is on successful paths. They are often categorized by the following types according to Parmenter (2010):

- Key result indicators (KRIs) show how a process can be done in a perspective or critical success factor.

- Result indicators (RIs) indicate what have been done.

- KPIs indidate what needs to be done towards established goals.

KPIs represent a set of measures focusing on the actions to improve the aspects of organizational performance that is the most critical for the current and future success of the organization. Each KPI has seven characteristics including:

(a) Non-financial measures (e.g., not expressed in dollars, yen, pounds, euros, etc.)

(b) Frequent records (e.g., 24/7, daily, or weekly)

(c) What actions taken by CEO and senior management team (e.g., CEO calls relevant staff to enquire what is going on)

(d) What actions taken by staff (e.g., staff can understand the measures and know what to fix)

(e) Measures that tie responsibility down to a team (e.g., CEO can call a team leader who can take the necessary action)

(f) Indicators that have signifiant impacts on performance

(g) Encouragement to appropriate actions for improvements in performance

(h) Patrik & Magnus (1999) also indicated dimensions and characteristics of manufacturing performance measures that are consistent with the above seven characteristics, except for the characteristic of simplicity which is suitable with SMEs' characteristics as well. The simplicity means the measure should be understandable and easy for data collections, calculations and reports.

Therefore, those characteristics should be taken into the selection of performance measures to have proper performance indicators. Overall equipment effectiveness (OEE), one of popular KPIs in manufacturing, is taken as an example to consider its compliance with the characteristics described by Table 1.

By taking the characteristics, Table 2 provides KPIs suggested for SMEs.

Table 1. Overall equipment effectiveness (OEE) and its characteristics

Characteristics	Description
(a)	OEE is an non-financial measure that gives a picture of performance taking availability rate (time utilization), performance rate, and quality rate into account.
(b)	OEE is normally measured in days, months, quarters, or years for showing the performance trend.
(c), (d), (e), (f), (g)	OEE is used by different enterprise levels, ranging from strategic to shop-floor levels. The top managers look at OEE to capture the overall effectiveness of whole factory so that they can make proper decisions, whereas the middle and operational levels find the OEE and its components (availability, performance rate, quality rate) as a directional compass for improvement and problem-solving priorities (Kashif et al., 2018).
(h)	OEE is a bottom-up method in which an integrated force is trained to maximize the equipment effectiveness (Amin & Fredrik, 2015). It is also a well-known application SMEs can make reference or benchmark.

Table 2. Characterized key performance indicators (KPIs) for small and medium-sized enterprises (SMEs)

Characterized KPIs for SMEs (x: the KPI was proposed by the according author(s))	Customer complaints	Supply on Time in Full	Stock loss (obsolete)	Productivity	Overall Equipment Effectiveness	Delivery on Time in Full	Environment, health, and safety (EHS) incidents
	KPI.1	KPI.2	KPI.3	KPI.4	KPI.5	KPI.7	KPI.8
Anagnostopoulos (2010)	x	x		x	x		
Bernard (2012)	x		x		x	x	
Carl-Fredrik et al. (2015)			x		x		
Enoch (2016)							x
Farzad & Kuan (2011)	x				x	x	
Henri et al. (2016)		x		x	x	x	
Kashif et al. (2018)					x		
Mourtzis (2015)	x	x	x		x		
Raymond & Pit-yan (2016)				x	x		
Sergei (2018)					x	x	

There are seven proposed KPIs that are suitable for SMEs to build a foundational manufacturing performance system. Nine out of ten research papers pointed out the OEE as a key performance measure whereas Enoch (2016) strongly proposed the incidents related to EHS as a safety KPIs in the manufacturing sector. They are linked together to create a package of KPIs as a starting point for SMEs regardless of manufacturing business sizes. Besides, the proposed KPIs can be managed by different business departments as the following proposal (Table 3).

By doing that, those enterprises (SMEs) which are lack of expertise and resources can easily set up the performance measurement foundation as well as practice it to get quickly experimental results before mass deployment or implementation of information technology solutions. However, in some special SMEs' business contexts where the SMEs also want to prioritize the KPIs so that they can focus their limited resources on top KPI priorities to the bottom. The solution for this is also the main contribution of the next part that presents the KPI priority with the application of

Table 3. Functional categorized key performance indicators (KPIs)

Functional KPIs	Unit	Business function
Customer complaints	#, %	Sales, marketing
Supply on Time in Full	%	Warehouse, inventory
Stock loss (obsolete)	$, %	Warehouse, inventory, accounting
Productivity	#	Production
Overall Equipment Effectiveness	%	Production, maintenance
Delivery on Time in Full	%	Production, quality, planning
Environment, health, and safety incidents	#, %	Safety, human resource

#, % and $ represent numeric, percentage, and finanial records respectively.

Table 4. Triangular fuzzy scale

Pair-wise Importance Scale								
Absolute	Very strong	Strong	Weak	Equal	Weak	Strong	Very strong	Absolute
9:1	7:1	5:1	3:1	1:1	1:3	1:5	1:7	1:9

fuzzy analytical hierarchy process (FAHP) whose technical inputs are given by the industrial experts.

2.2. The methodology of FAHP for prioritizing KPIs

Every business process has its own management goals and objectives that are ideally written in KPIs in compliance with SMART criteria (specific, measurable, attainable, realistic, and time-related) to avoid the risks that they could be unachievable (Doran, 1981). The evaluation was done by the group of three experts, who have strong experience in the field of operational excellence and production management. They will evaluate and prioritize each KPI based on pair-wise comparison towards SMART criteria.

The pair-wise comparison can be done by the analytical hierarchy process (AHP) proposed by Arash & Mahbod (2007). However, the AHP method may contribute to the imprecise judgments of decision makers, which can be improved by the application of FAHP (Aşkın & Güzin, 2007). In addition, FAHP can reduce or even eliminate the fuzziness; vagueness existing in many decisions made by multiple makers (Ali & William, 2018).

Therefore, evaluating each proposed KPI with the SMART principle in combination with FAHP to prioritize them will be a comprehensive package of KPIs that suits with the SMEs' different contexts. The FAHP model is represented by triangular fuzzy numbers that are identified as triple M = (l, m, u) in which l, m, and u stand for the lower, medium and upper values of M, respectively ($l \leq m \leq u$). Its function is defined as (Chang, 1996) :

$$\mu_{\mathrm{M}}(x) = \begin{cases} \dfrac{x}{\mathrm{m}-\mathrm{l}} - \dfrac{\mathrm{l}}{\mathrm{m}-\mathrm{l}}, & x \in [\mathrm{l}, \mathrm{m}] \\ \dfrac{x}{\mathrm{m}-\mathrm{u}} - \dfrac{\mathrm{l}}{\mathrm{m}-\mathrm{u}}, & x \in [\mathrm{l}, \mathrm{m}] \\ 0, & \text{otherwise} \end{cases}$$

Table 4 is used as the measurement scale of the triangular fuzzy model:

The first step in the FAHP process is to structure the hierarchy of KPIs with SMART criteria,which is described by Figure 1.

The pair-wise comparison is conducted on both levels in which level 1 is a pair-wise comparison of SMART criteria with each other in terms of SME's manufacturing performance system evaluated by the three experts. Subsequently, level 2 is also a paire-wise comparison of among KPIs towards each criterion of SMART principle.

Specifically, each expert will be asked to grade the importance of one sub-criterion over another on the same level with respect to the top criterion as the extracted part of the survey provided by Table 5. According to the expert with the survey below, the "specific" criterion is equally important as the "measurable", but less important than the "assignable" characteristic in terms of manufacturing performance system. That means as the construction of manufacturing performance system, the SMEs should consider the "assignable" characteristic of a KPI.

After getting the inputs from the group of industrial experts, the data was analyzed accord-

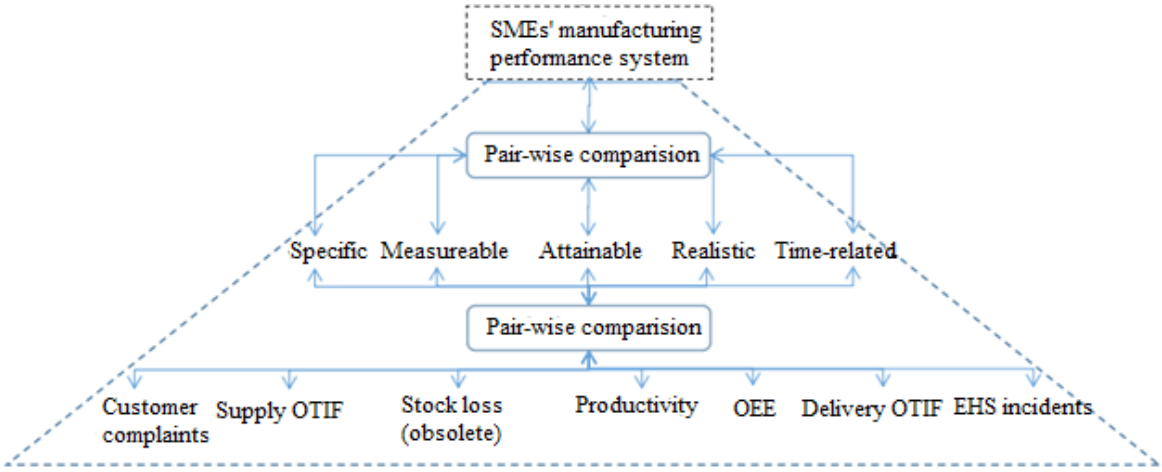

Figure 1. Hierarchy tree for fuzzy analytical hierarchy process pair-wise comparison.

Table 5. An extracted part of the survey

	Specific	Measurable	Assignable	Realistic	Time-related
Specific		1	-2	2	3
Measurable			-2	1	3
Assignable				2	2
Realistic					2
Time-related					

Based on your expertise, please grade the importance of each SMART criterion over others with respect to SMEs' manufacturing performance system based the triangular fuzzy scale.

Table 6. Average consistency ratio (CR) of first level

Average of consistency ratio (CR)	Specific	Measurable	Attainable	Realistic	Time-related
SMEs' manufacturing performance system			0.037		
Key performance indicators	0.080	0.077	0.077	0.088	0.097

ing to the procedure proposed by Amy et al. (2009) with the testing results of consistency in the response of the experts (Table 6). The consistency ratio for both levels show the suitability of the FAHP model for the data inputs due to its value is below then the CR validation value of 0.1. Therefore, the following weights for each KPI with respect to SMEs' manufacturing performance system indicate the KPI prioritization by which the SMEs can focus their limited resources on the implementation instead of mass deployment.

Table 7 shows the result of FAHP analysis indicating the rank of KPI importance from the point of views given by the experts. The most high-ranking KPI is OEE whose calculated weight is 0.223 whereas that of *stock loss* is the lowest one with the weight of 0.036. Based on the result, SMEs should kick off the implementation of those KPIs according to the prioritization that suits their business context. By measuring OEE, the efficiency and effectiveness of a manufacturing workstation, including one or more operators and machines, are identified. Based on the current workstation performance, the improvement actions can be brainstormed and focused on weaknesses represented by the lowest percentage of OEE components (availability, performance, and quality). There are also some popular lean techniques to increase OEE, such as single minute exchange of dies (Andreia & Alexandra, 2010), or design of experiment (Anand & Nandurkar, 2012). These methods will bring significant insights of improvement opportunities for manufac-

Table 7. Key performance indicator (KPI) ranking with respect to small and medium-sized enterprises' manufacturing performance system

KPI prioritization	Description	Weights
1	Overall equipment effectiveness	0.223
2	Customer complaints	0.198
3	Productivity	0.196
4	Delivery on time in full	0.155
5	Supply on time in full	0.149
6	Environment, health, and safety incidents	0.043
7	Stock loss (obsolete)	0.036

turing performance.

Another source for exposing the opportunities for improvements is the *customer complaints* which require the SMEs to have the analysis of failure or root cause for the problems in accordance with the corrective actions. The standard procedure should follow ISO 9001 standards as minimum requirements and the reports must be recorded as the lessons learned to avoid the repetitive problems or noncompliance.

With the measurement of *productivity*, its trend not only shows how much the SMEs should put effort for improving the productivity but also alarm how the customer order can be achieved by capacity investment or continuous improvements. At the end of the day, the productivity matters the most due to the fact that the output rate per production time unit or headcount shows how well the manufacturer minimizes its resources to maximize the output, which in turn satisfies the customer order by delivery in time on full qualified products.

What the customer needs is not just only the full quantity with agreed cost but the order must be available at the right place at the right time, where the concept of just in time (JIT) was born (Gupta & Garg, 2012). Its KPI should be measured in percentage, frequently monitored, and set up target of 100% orders are *delivered on time in full*. Additionally, Kanban which is one of the JIT tools can be adapted by SMEs to improve the KPIs by enabling both internal and external delivery processes to work smoothly with least waste, least work in progress (WIP) and lead time (Abdul et al., 2013).

By looking back to the upstream supply chain, the requirements of SMEs to their sub-suppliers are quite similar with the customers' point of view. Not only must the quality be met, but the sub-suppliers have to *supply the input materials on time with the right quantity and quality*. Their performance should be managed in form of percentage with the frequent data records of the supply compliance and process audit. By doing that, the production schedule can be guaranteed without negative effects due to lack of materials or non-compliance material quality.

Not to mention SMEs' operational performance, the increasing awareness of EHS across the large international enterprises pushes the prominent requirements of EHS compliance on SMEs (Kim, 2007). Therefore, in order to increase the chance of joining the global supply chain SMEs need to meet EHS compliance standards required by sourcing enterprises. The KPI of EHS incidents is an approachable starting point for those who are lack of resources in pursuing the international standards, like ISO 140001 for environment or OHSAS 18001 for occupational health and safety, to name just a few.

Finally, the *stock loss* points out the lack of material flow management in which both input materials and finished products could be lost or obsoleted, resulting in major financial loss. Due to lack of resources in implementing the management software, like enterprise resource planning, the KPI is easily implemented for SMEs in combination with frequent accounting audits during a year. By keeping the data on track, the SMEs will be alarmed to have immediate corrective actions before stock major losses.

At this stage, the next step for SMEs to successfully implement the performance system is to brainstorm a comprehensive road map in which the suitable tools for data collection, performance tracking and displays, report interpretation, communication flow across the staff levels must be determined. The final part will show some guidelines that fits SMEs' context.

Figure 2. Two-way communication flow of performance system.

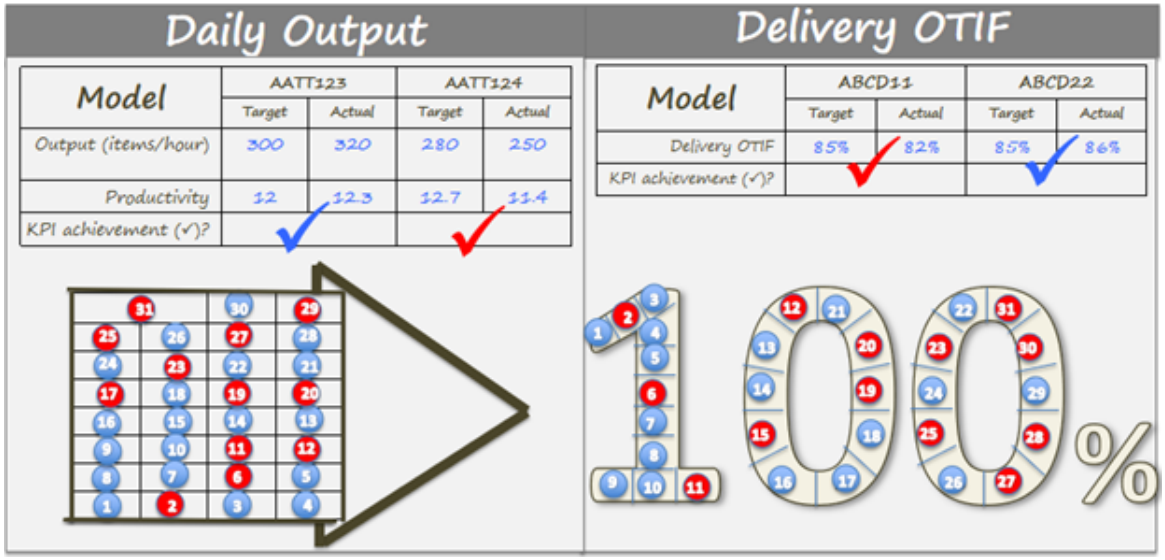

Figure 3. Visual record and display for key performance indicator (KPI) communication.

3. Results and Discussion

No matter what system SMEs are going to implement, the commitment from the managerial levels plays a decisive role on the success. The commitment must be translated into business actions from the top management levels to operational ones; specifically, the performance management system has to be communicated to the entire organization as the two-way communication flow (Figure 2).

Figure 2 shows that the commitment can be proved as frequent meetings throughout the organization by grouping different cross-functional or working levels together so that they can feel the importance of work, keep on track the progress, as well as increasing the responsibility of the staff. During the meetings, the KPIs are the main topics for discussion on how improvements can be made, which will also improve unintendedly the employee morale due to the scene of free-speaking ideas.

To make the communication flow smoothly, the SMEs should have tools for supporting the record of data as simplification as possible as it comes to operational levels, like operators who normally don't have many opportunities to learn and use the complex procedure or system. Therefore, the

most approachable way is to apply visual display with some cost-effective accessories like the table or handbook as Figure 3.

As can be seen by the figure, the simple visual method does not require any special understanding in technical terms (Nguyen et al., 2017), but indeed it communicates easily to all about the performance status. Described by Figure 3, the staff will be notified as the failure in the corresponding KPI with the red-highlighted dots whose numbers inside indicate the days of the month. Based on the alerts, the supervisor and its responsible members will brainstorm the root causes and then preventive actions. Finally, these activities must be recorded in document and the best solution is to follow the ISO 9001 standards in a real sense.

4. Conclusions

To enhance the competitiveness and join the global value chain, SMEs have no ways but make their operations themselves toward excellence. One of the critical steps is to develop and implement the performance system. By taking account the inherent weakness of SMEs who are mostly lack of resource and expertise to deploy such systems, the paper provides seven important KPIs to measure its manufacturing performance. Besides, the paper takes one step further to prioritize these KPIs based on the industrial experts' experience with high quality outcome by applying the FHAP. Therefore, the SMEs should consider firstly OEE as a key KPI for the experiment if needed and then apply the rest in order to avoid spending much effort.

Last but not least, the system should be deployed in a practical approach with the commitment from top management by conducting clear and quick-win meetings across working levels to make sure all the staff are on the same page.

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Conflicts of interest

The authors declare no conflicts of interest.

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The effects of plant spacing on yield and quality of butterfly pea (*Clitoria ternatea* L.) cultivated in organic-oriented farming system on grey soil

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ABSTRACT

Clitoria ternatea L. is a plant species that can be used in food and pharmaceutical industry. This study was conducted to evaluate the effects of different plant spacing on the productivity and quality of butterfly pea grown on grey soil in Thu Duc, Ho Chi Minh City. Six treatments correspond to plant spacing of 80 x 15 cm, 80 x 20 cm, 80 x 25 cm, 80 x 30 cm, 80 x 35 cm and 80 x 40 cm. The results showed that the highest flower amount (296.8 flowers/plant) was obtained with butterfly pea planted at the spacing of 80 x 15 cm, commercial flower weight (7.86 g/100 flowers), theoretical yield of fresh flower (1,779.0 kg/1,000 m^2), actual yield of fresh flower (841.9 kg/1,000 m^2), theoretical yield of commercial flower (194.6 kg/1,000 m^2) and actual yield of commercial flower (89.0 kg/1,000 m^2). Nevertheless, plant spacings did not affect the dry matter ratio, anthocyanin and tannin content in the commercial butterfly flowers.

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1. Introduction

Clitoria tenatea L. also known as butterfly pea, is a species belonging to Fabaceae family. Currently, the flowers from butterfly pea are being used in food, medicine as well as in cosmetics (Morris, 2009). Especially, the dried butterfly pea flowers can also be used as tea. Butterfly pea tea is characterized by a rich source of natural antioxidants (Kamkaen & Wilkinson, 2009), which is also highly safe (Luu, 2005) and satisfied most requirements of the consumers. Consequently, the flowers are consumed increasingly as healthy food. However, the research on farming techniques which are necessary for butterfly pea reaching high yield and quality, are still limited.

Plant spacing is an important determinant of plant growth, development and productivity (McMurray, 2004; McRae et al., 2008; Khaliq et al., 2009). The impact of crop density is mainly due to differences in solar radiation distribution. An optimization of solar radiation uptake is the most important for photosynthetic efficiency. An appropriate plant density or spacing helps plants take advantage of sunlight energy, reducing pests and diseases, paving the way for high productivity. In addition, appropriate spacing can also save the seedlings, labor and other costs, those turn out to improve the economic efficiency. Contreras et al. (2012) concluded that when planting but-

terfly pea plant with a distance of 25 x 25 cm gave highest total grain yield, grain yield per plant, number of pods per plant, number of pods per m^2, fruit length, number of seeds in pod as well as the seed weight. However, there is no recommendation for suitable plant spacing for butterfly pea flower used as tea. Therefore, the aim of this research was to identify suitable plant spacing for butterfly pea growing on grey soil in organic-oriented farming system.

2. Materials and Methods

2.1. Experimental design

The experiment was conducted at the Experimental field of Faculty of Agronomy, Nong Lam University, Ho Chi Minh City (September 2019 to January 2020).

The seeds of double-winged butterfly pea variety (collected in Pham Van Coi Commune, Cu Chi District, Ho Chi Minh City) was sown.

Nutrient used for the whole experiment was well composed cow dung, that supplied by the Experimental field of Animal Science and Veterinary Medicine Faculty, Nong Lam University in Ho Chi Minh city. The manure was applied at a rate of 5.0 tons/ha at 15 days before planting.

Insects and butterflies occurrences on the experimental field were cached manually. No other chemical fertilizers or pesticides was used before and during cultivation period.

One-factor experiment was arranged in a Randomized Completed Block Design (RCBD) included 6 treatments with 3 replicates. The plant spacing in the experiment included: 80 x 15 cm (8,333 plants/1,000 m^2), 80 x 20 cm (6,125 plants/1,000 m^2), 80 x 25 cm (The control) (5,000 plants/1,000 m^2), 80 x 30 cm (4,167 plants/1,000 m^2), 80 x 35 cm (3,571 plants/1,000 m^2) and 80 x 40 cm (3,125 plants/1,000 m^2). Total number of experimental plots was 18 plots; a single plot area was 16.8 m^2; The spacing between two neighbouring plots was 0.5 m; The whole experimental area was 302.4 m^2.

2.2. Land preparation and field management

Beds were established with a size of 6.0 x 2.8 m, each bed consisted of 3 single rows, each bed was 80 cm apart and 20 cm from the aisle; Composted cow dung was applied at the rate of 5 tons/ha 15 days before planting.

Staking setup: U-shaped staking was made of bamboo, with a height of 1.5 m, each row consisted of 7 bamboo poles separated by 1.0 m; a black film was used to cover along the rows for weed preventing.

Seeds were sown on a nursery. After 15 days, the seedlings reached 3 pairs of leaves which then were transplanted onto the experimental field. At 60 days after planting, the plant tips were cut off for branch boosting. Experimental field was watered once/day. Weed control was conducted manually once in every 15 days.

When butterfly pea at flowering stage, new opening flowers were harvested every 2 days. The fresh flowers were left on open air in 48 hours for naturally dryness, then finally, dried at 95°C for 40 minutes (Luong, 2004).

2.3. Data collection and statistics

Data from following parameters were collected, including:

Total numbers of flowers per plant (flowers/plant): Count the average number of flowers on the target plants at all harvests until the end of the experiment; Fresh flowers weight (g/100 flowers): Weigh 100 fresh flowers at harvest time 60, 75 and 90 days after planting, then average; Commercial flower weight (g/100 flowers): Weigh 100 dried flowers at harvest time 60, 75 and 90 days after planting, after drying at 95°C for 40 minutes and then average; Theoretical fresh flower yield (kg/1,000 m^2): [Total number of flowers/plant (flower/plant) * fresh flower weight (g/100 flowers) * number of plants/1,000 m^2]/105; Actual fresh flower yield (kg/1,000 m^2): [Total fresh flowers weight/plot (kg) x 1,000]/16,8; Theoretical commercial flower yield (kg/1,000 m^2): [Total number of flowers/plant (flower/plant) * commercial flower weight (g/100 flowers) * number of plants/1,000 m^2]/105; Actual commercial flower yield (kg/1,000 m^2): [Total commercial flowers weight/plot (kg) x 1,000]/16,8.

Anthocyanin content in commercial flowers was determined using the method TCVN 11028:2015; Tannin content was determined by Leventhal method.

Data analysis was conducted with ANOVA test and Duncan rank at significance level $\alpha = 0.01$ using SAS 9.1 software.

Table 1. Soil characters of the experimental plots[1]

Indices	Unit	Result	Method
$pH_{KCl_{(1:5)}}$		5.501	pH meter
$EC_{(1:5)}$	mS/cm	0.367	EC meter
Total Organic Carbon	%	0.718	Tiurin
Total N	%	0.062	Kjeldahl
N-NH_4^+	mg/100 g	0.597	Devardar – Alloy
C/N		11.581	
Total P_2O_5	%	0.053	Colorimeter
Availability P_2O_5	mg/100 g	4.390	Bray #1
Total K_2O	%	0.154	Flame photometer
Exchangeability K_2O	mg/100 g	5.085	Flame photometer
CEC	meq/100 g	7.801	Acetate Amonium
Soil texture	%	Sand: 82.20 Loam: 13.05 Clay: 4.75	Densitometrer

[1]Analyzed by Department of Soil Science, Faculty of Agronomy, Nong Lam University, 2019.

Table 2. Amount and mass of butterfly pea flower under the influence of plant spacings

Plant spacing (cm)	Flower amount (flowers/plant)	Fresh flower weight (g/100 flowers)	Commercial flower weight (g/100 flowers)
80 x 15	296.80^a	73.23^{ab}	7.86^a
80 x 20	290.70^a	73.98^a	7.88^a
80 x 25 (Control)	277.23^a	72.95^{ab}	7.92^a
80 x 30	249.20^b	70.20^b	7.22^b
80 x 35	204.50^c	70.18^b	7.33^b
80 x 40	180.00^c	69.95^b	7.46^b
CV (%)	4.27	1.67	1.48
F value	60.93**	6.95**	22.77**

$^{a-c}$In the same column, numbers with the same character are statistically insignificant difference.
**: the difference is statistically significant at $\alpha = 0.01$.

3. Results and Discussions

3.1. Evaluation of soil quality at the experimental field

Physical and chemical analysis results of the experimental soil (Table 1) suggested that the experiment plot soil has texture containing 82.20% of sand, 13.05% of loam and 4.75% of clay. According to García-Gaines & Frankenstein (2015) the soil at the experimental field is belonging to loamy sand texture. The soil was highly acidic ($pH_{KCl_{(1:5)}}$ = 5.501) and not saline ($EC_{(1:5)}$ = 0.367 mS/cm) (Slavich & Petterson, 1993). It was recommended that the soil pH ranged from 5.5 to 8.9, which was acceptable for butterfly pea (Singh et al., 2017).

The soil organic C content was low (0.718%) and the C/N ratio was 11.581. The soil had low levels of macronutrients (Rayment & Lyons, 2011). Furthermore, cation exchange capability was also low. However, butterfly pea is a native plant, it is highly adaptable to various soil types therefore this location was acceptable for butterfly pea cultivation. Even those, organic fertilizer supplement is necessary to provide nutrients for plants during cultivation.

3.2. Influence of plant spacing to amount and mass of butterfly pea flower

The number of flowers and flower weight are most important factor correlating to butterfly pea flower yield. At the same plant spacing, the

Table 3. Theoretical and actual yields of butterfly pea under the influence of plant spacing

Plant spacing (cm)	Theoretical fresh flower yield (kg/1000 m^2)	Actual fresh flower yield (kg/1000 m^2)
80 x 15	1,812.6[a]	841.9[a]
80 x 20	1,345.5[b]	721.9[b]
80 x 25 (Control)	1,011.0[c]	562.2[c]
80 x 30	728.5[d]	511.0[c]
80 x 35	511.8[e]	442.3[cd]
80 x 40	395.0[e]	371.6[d]
CV (%)	6.3	7.7
F value	233.9**	47.3**

[a-e]In the same column, numbers with the same character are statistically insignificant difference.
**: the difference is statistically significant at $\alpha = 0.01$.

greater the number of flowers and the heavier weight, the higher the yield will be. Results presented in Table 2 showed that the total number of flowers per plant was significantly different between plants grown at different spacing in the experiment. Planting at the spacing of 80 x 15 cm gave the most flowers (296.8 flowers/plant), but not statistically different from the plant spacing of 80 x 20 cm (290,70 flowers/plant) and 80 x 15 cm (277.23 flowers/plant). Planting at the spacing of 80 x 40 cm obtained lowest number of flowers (only 180 flowers/plant), the difference was 116.80 flowers/plant lower as compared to planting at the spacing of 80 x 15 cm.

Fresh flower weight and commercial flower weight of butterfly pea plants were significantly different under the influence of different plant spacing. Butterfly pea plants grown at a spacing of 80 x 20 cm gained the highest fresh flower weight (73.98 g/100 flowers), event it was not statistically different from planting at the spacing of 80 x 15 cm or 80 x 25 cm. The lowest fresh flowers weight gained when planting at the spacing of 80 x 40 cm (69.95 g/100 flowers). It was 4.03 grams lower than when planting at the spacing of 80 x 15 cm.

Similarly, the highest commercial flower weight of butterfly pea was obtained when planting at the spacing of 80 x 20 cm (7.88 g/100 flowers) even it was not statistically different from planting at the spacing of 80 x 15 cm or 80 x 25 cm. Planting at the spacing of 80 x 40 cm gained lowest commercial flower weight (7.46 g/100 flowers) but which was not statistically different from planting at the spacing of 80 x 30 or 80 x 35 cm; It was only 0.42 g lower if compared to planting at spacing of 80 x 15 cm.

Because the experiment conducted at the end of rainy season, most of growth season was during dry and hot weather, plant population at higher density perhaps made microclimate not as hot as in lower density population. The result shown that at higher plant spacing (80 x 15 cm, 80 x 20 cm, 80 x 25 cm), butterfly pea plants grown better, giving more flowers and higher flower mass. This result were in accordance with a report by Tran & Pham (2018) on *Limnophila rugosa* (Roth) Merr. when they found that growing in a long spacing, especially when the growing substrate covered so it is less affected. Because of high temperature at the experimental area, the plants grew well and formed more leaves.

3.3. Influence of plant spacing to theoretical and actual yields of butterfly pea

Results presented in Table 3 shown that butterfly pea grown at the spacing of 80 x 15 cm reached highest theoretical yield as well as actual flower yield (1,812.6 and 841.9 kg/1,000 m2, respectively). The actual yield of fresh flowers accounts for 46.4% of the theoretical fresh flower yield. Besides, butterfly pea grown at the spacing of 80 x 40 cm, yielding the lowest theoretical and actual fresh flower yields (395.0 and 371.6 kg/1,000 m^2, respectively). The actual yield of fresh flowers accounts for 94.1% of the theoretical fresh flower productivity. The difference in the ratio of actual yield and theoretical yield was due to the level of coverage of the pea plants. When the pea plants were higher density, it might lead to the plants being overlapped. In fact, plants at high density tended to be overlapped each other, which affected to actual numbers of harvestable flowers on the plot. As a consequence, there was a big difference between the theoretical fresh yield

Table 4. Commercial flower yields of butterfly pea under the influence of plant spacings

Plant spacing (cm)	Theoretical commercial flower yield (kg/1000 m^2)	Actual commercial flower yield (kg/1000 m^2)
80 x 15	194.6[a]	89.0[a]
80 x 20	143.4[b]	75.9[b]
80 x 25 (Control)	109.8[c]	60.1[c]
80 x 30	75.0[d]	52.5[cd]
80 x 35	53.6[e]	45.7[de]
80 x 40	42.0[e]	39.0[e]
CV (%)	5.7	7.3
F value	296.6**	55.2**

[a-e]In the same column, numbers with the same character are statistically insignificant difference.
**: the difference is statistically significant at $\alpha = 0.01$.

Table 5. Dry matter, anthocyanin and tannin contents of butterfly pea flowers under influence of plant spacings

Plant spacing (cm)	Contents (%)		
	Dry matter	Anthocyanin	Tannin
80 x 15	10.753	0.538	1.817
80 x 20	10.661	0.546	1.824
80 x 25 (control)	10.856	0.538	1.820
80 x 30	10.295	0.540	1.824
80 x 35	10.490	0.540	1.827
80 x 40	10.635	0.536	1.829
CV (%)	1.930	1.745	0.483
F value	2.831[ns]	0.380[ns]	0.760[ns]

ns: non-significant.

and the actual yield. Less shading between plants reduced the difference.

3.4. Influence of plant spacing to commercial flower yields of butterfly pea

Commercial flower ratio is a determinant of economic efficiency for the farmers. In this research, butterfly pea flowers were naturally dried for 48 hours at room temperature then transferred to temperature of 95°C for 40 min in order to reach commercial quality level with moisture content was about 12%. The results presented in Table 4 shown that the difference of theoretical and actual commercial flower yield was statistical significance between flower collected from plants grown in different spacing. Butterfly pea plants grown at the spacing of 80 x 15 cm obtained the highest theoretical commercial yield (194.6 kg/1000 m^2); the plants grown at the spacing of 80 x 40 cm (42.0 kg/1000 m^2), was 152.6 kg/1000 m^2 lower than that.

The actual harvest of commercial flowers was also highest with the butterfly pea planted at the spacing of 80 x 15 (reaching 89.0 kg/1,000 m^2). It was statistically significant higher as compared to plants in all other treatments. Plant grown at the spacing of 80 x 40 cm, showed the lowest commercial flower (39.0 kg/1,000 m^2); it was 40 kg/1,000 m^2 lower as compared to plants grown at the spacing 80 x 15 cm.

In general, it was obvious that the plant density greatly affected to both theoretical and commercial flower yields. The butterfly pea when grown at a higher density obtained a higher yield. At the same time, the difference between theoretical yield and actual yield was high.

3.5. Influence of plant spacing to dry matter, anthocyanin and tannin contents of butterfly pea flowers

Results in Table 5 indicated that different plant spacing did not affect the quality of butterfly pea flower indices including dry matter ratio, anthocyanin and tannin content in dried flowers. The dry matter ratio of the butterfly pea flower ranged from 10.3 to 10.86%. In the commercial

flowers, anthocyanin content ranged from 0.54 to 0.55%. This result suggested that anthocyanin in butterfly pea flowers is higher than that in some fruits such as blueberries (0.08 to 0.53%), cherry (0.35 to 0.45%), black raspberry (0.08 to 0.18%) (Horovitz et al., 2008). Anthocyanin related to the intensity of plant colour, the darker the colour, the higher the anthocyanin content. Nevertheless, tannin content in commercial butterfly peas ranged from 1.82 to 1.83%, is much lower than that in black tea (13.36%), green tea (2.65%) and Oolong tea (8.66%) (Khasnabis et al., 2015). Tannin is polyphenol compounds in plants that help to against bacteria and induce acrid taste, it plays an important role in the quality of tea products.

4. Conclusions

Pea flower of plants grown at the spacing of 80 x 15 cm gained highest number of flowers on plant (296.8 flowers/tree), dry flower weight (7.86 g/100 flowers), the theoretical fresh flower yield (1,779.0 kg/1,000 m^2), the actual fresh flower yield (841.9 kg/1,000 m^2), the theoretical commercial flower yield (194.6 kg/1,000 m^2) as well as the actual commercial flower yield (89.0 kg/1,000 m^2). The different plant spacing did not affect quality criteria such as dry matter, anthocyanin and tannin content in commercial butterfly pea flower.

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Antibiotic sensitivity of common respiratory bacteria of pig from Hubei province, China

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ABSTRACT

The use of antimicrobials for feeding and treatment is crucial to animal health. However, continuous use of antibiotics is contributing to emergence and widespread of antibiotic resistance. This study aimed to investigate the antimicrobial resistance of five major respiratory pathogens in pigs of Hubei province, China, from October to December, 2019. Antibiotic susceptibility testing for *Streptococcus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, *Bordetella bronchiseptica* and *Actinobacillus pleuropneumoniae* was determined to representatives of relevant antibiotic classes.

Streptococcus suis isolates were mostly sensitive to beta-lactams, whereas high levels of resistance were observed to quinolones, gentamycin, doxycycline, trimethoprime and lincomycin. For *H. parasuis*, *P. multocida* and *A. pleuropneumoniae* of *Pasteurellaceae* family, the susceptibility to beta-lactams and quinolones was displayed. Most *B. bronchiseptica* isolates were sensitive to doxycycline, azithomycin, polymycin whereas high resistance levels to beta-lactams, aminoglycosides and quinolones were recorded.

This study obtained practical data for later studies and usage to combat infections due to respiratory bacteria.

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1. Introduction

Porcine respiratory diseases complex is caused by multifactorial aetiologies, including the viral and bacterial pathogens, the environment, management and genetic factors. Within this complex, *Streptococcus suis*, *Haemophillus parasuis*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Bordetella bronchiseptica* have been known to be ubiquitous in almost all pig farms. *S. suis* is as a major respiratory commensal and pathogen of pigs and an emerging zoonotic agent of meningitis in human (Goyette-Desjardins et al., 2014). *Haemophillus parasuis* produces Glässer's disease as well as pneumonia (Nedbalcova et al., 2006). *Pasteurella multocida* causes atrophic rhinitis, particularly when combined with *B. bronchiseptica* (Jeffrey et al., 2013). *Actinobacillus pleuropneumoniae* generates contagious hemorrhagic pleuropneumonia in pigs (Brownfield, 2013). Due to their complexity and indeterminacy, bacterial diseases are very challenging to control.

Antimicrobial agents are important for effective production of food animals as growth promoter or/and disease prevention. As the world's largest pork producer and consumer, China has been reported for the massive use of antibiotic in food animal production. Zhao et al. (2011) showed antimicrobial susceptibility tests on *B. bronchiseptica* isolates from Chinese farms that were highly resistant to ampicillin, cefazolin,

streptomycin, amoxicillin and tetracycline. Zhang et al. (2015) found the most antibiotics consumed in China's swine farming were fluoroquinolones and β-lactams. Therefore, antimicrobial surveillance is necessary to provide a better understanding of antibiotic resistance in the animal population.

This study aimed to contribute the comprehension of the antibiotic susceptibility pattern of *S. suis*, *H. parasuis*, *P. multocida*, *A. pleuroplneumoniae* and *B. bronchiseptica*, the five important pathogens found in the respiratory tract of pigs in Hubei province, China, using disk diffusion test.

2. Materials and Methods

2.1. Sample collection

From October to December 2019, a total of 155 samples from 14 different pig farms in Hubei province were sent to the Animal Diagnostic Center of Huazhong University. The collected samples included lungs, spleen, synovial fluid, brain, tracheal effusion etc. Lived pigs were observed for evaluating clinical signs and endured necropsy to collect samples. For every individual pig, lung and spleen samples were sealed in a clean zipper bag; brain and synovial fluid were kept in an eppendorf tubes (EP tube). Nasal samples were collected by using sterile cotton swabs and placed in sterilized EP tubes. The samples were clearly marked.

After the period of three months, 133 strains of the five concerned bacteria species from 155 samples were isolated and identified by using multiplex PCR assays. For the identification of the five bacteria, the primers of following target genes were used: 16S rRNA to detect *S. suis* (Cheung, 2008), 16S rRNA for *H. parasuis*, apxIV for *A. pleuropneumoniae*, fla for *B. bronchiseptica* (Xue, 2009) and ktm1 for *P. multocida* (Nagai et al., 1994). The greatest number of isolated strains were obtained from *S. suis* (40%, 62/155), followed by H. parasuis (18.71%, 29/155), *P. multocida* (14.83%, 23/155), *B. bronchiseptica* (8.39%, 13/155), and *A. pleuropneumoniae* (3.87%, 6/155).

2.2. Kirby-Bauer antibiotic testing

Twenty antibiotic agents (Hangzhou Binhe Microorganism Reagent Co., Ltd) were used, including cefotazime (30 µg), cephradine (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), amoxicillin (20 µg) and ampicillin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg), norfloxacin (10 µg), spectinomycin (100 µg), gentamicin (10 µg), streptomycin (10 µg), amikacin (30 µg), kanamycin (30 µg), doxycycline (30 µg), lincomycin (30 µg), azithromycin (15 µg), polymyxin B (300 µg) and trimethoprim (23.75/1.25 µg).

Each purified isolates of tested bacteria were evenly spread onto a tryptic soy agar plate (TSA, BD^{TM}, USA) that had been coated with nicotinamide adenine dinucleotide liquid (NAD, Guangzhou Saiguo Biotech, China) and bovine serum (Zhejiang Tianhang Biotechnology, China). The antimicrobial discs were placed onto the surface of the agar. The plates were then incubated at 37°C for about 24 h. The inhibition zone diameter was measured and compared with standardized CLSI interpretive criteria to designate the isolate as sensitive, intermediate or resistant to the drug (CLSI, 2018). In this study, the isolates that showed intermediate were classified as resistant.

2.3. Results and Discussion

The resistant and sensitive rates of the five bacteria species to 20 antibiotic agents are presented in Table 1. Results showed the resistance rates of *S. suis* strains to quinolones, aminoglycosides, macrolides, lincomycins, tetracyclines, polymyxins and sulfonamides were all over 60%. *H. pasasuis* strains were sensitive to majority of the drugs but highly resistant to amoxicillin, streptomycin, amikacin, kanamycin and lincomycin. The resistance of *P. multocida* strains to aminoglycosides and lincosamides were apparently high compared to other antibiotic groups (Table 1).

With the small number of isolates being tested, the two purified isolates of *A. pleuropneumoniae* were sensitive to beta-lactams, quinolones and aminoglycosides. In contrast, all of the three isolates of *B. bronchiseptica* resisted to those drugs and only sensed to doxycycline, gentamicin, azithromycin and polymyxin B.

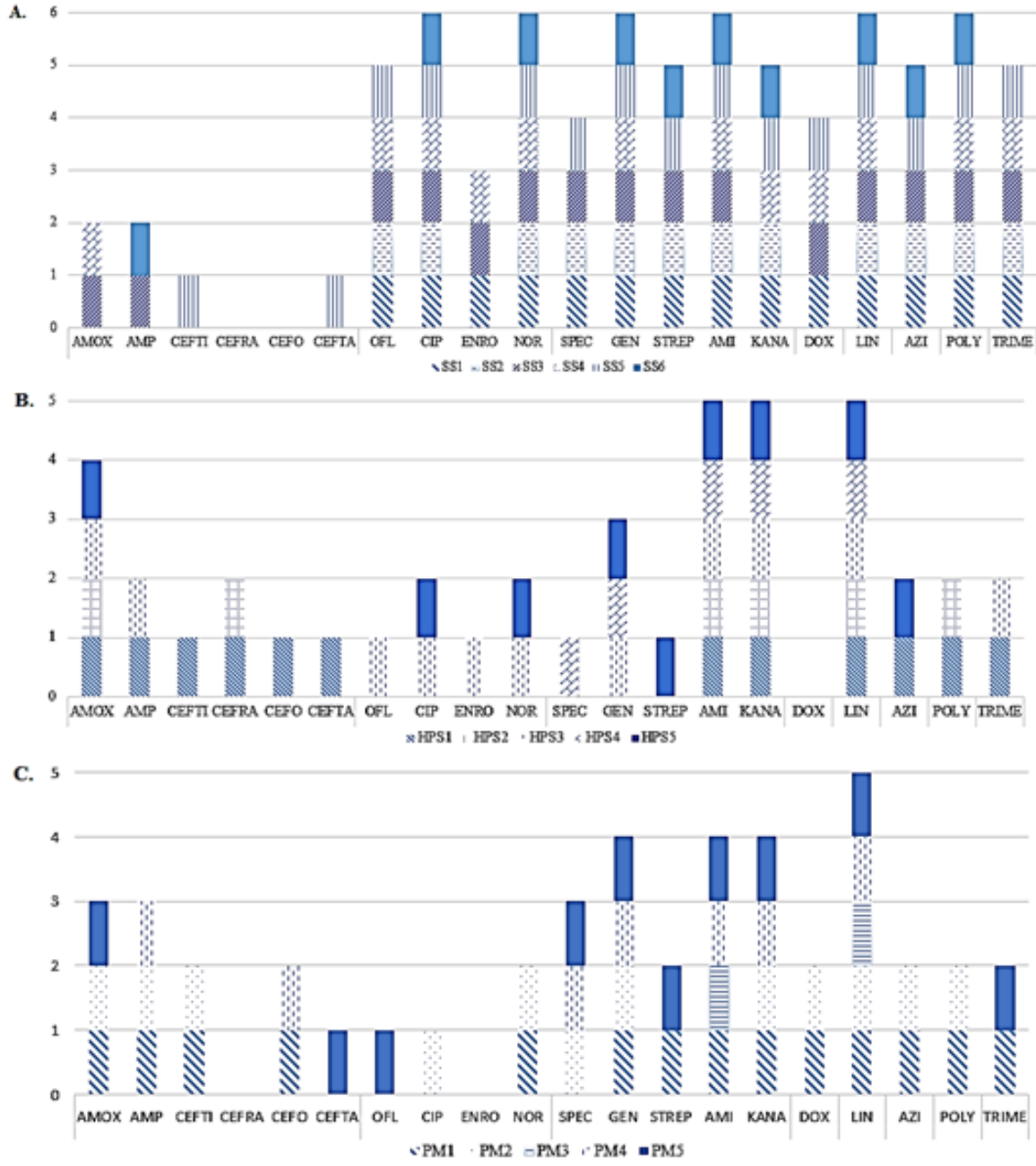
The drug-resistance pattern of bacterial isolates obtained in this study indicates that *S. suis*, *H. parasuis*, *P. multocida*, *B. bronchiseptica* and *A. pleuropneumoniae* displayed high antibiotic resistance rates to 8 tested antibiotics/antimicrobial classes. The resistance proportion of *S. suis* to these antibiotics were all

Table 1. Antibiotic susceptibility rates (%) and number of *S. suis*, *H. parasuis* and *P. multocida* isolates (in brackets) from infected pigs of Hubei province

Antibiotics	*S. suis*		*H. pasasuis*		*P. multocida*	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Amoxicillin	62.5 (5)	37.5 (3)	20.0 (1)	80.0 (4)	25.0 (1)	75.0 (3)
Ampicillin	75.0 (6)	25.0 (2)	50.0 (2)	50.0 (2)	40.0 (2)	60.0 (3)
Ceftiaxone	75.0 (6)	25.0 (2)	80.0 (4)	20.0 (1)	60.0 (3)	40.0 (2)
Cefotaxime	100.0 (6)	0.0 (0)	75.0 (3)	25.0 (1)	50.0 (2)	50.0 (2)
Ceftazidime	25.0 (1)	75.0 (3)	66.7 (2)	33.3 (1)	0.0 (0)	100.0 (1)
Cefradine	80.0 (4)	20.0 (1)	60.0 (3)	40.0 (2)	100.0 (4)	0.0 (0)
Ofloxacin	25.0 (2)	75.0 (6)	80.0 (4)	20.0 (1)	80.0 (4)	20.0 (1)
Ciprofloxacin	0.0 (0)	100.0 (8)	50.0 (2)	50.0 (2)	80.0 (4)	20.0 (1)
Enrofloxacin	50.0 (4)	50.0 (4)	75.0 (3)	25.0 (1)	100.0 (4)	0.0 (0)
Norfloxacin	0.0 (0)	100.0 (8)	60.0 (3)	40.0 (2)	60.0 (3)	40.0 (2)
Spectinomycin	37.5 (3)	62.5 (5)	80.0 (4)	20.0 (1)	40.0 (2)	60.0 (3)
Gentamicin	0.0 (0)	100.0 (8)	40.0 (2)	60.0 (3)	20.0 (1)	80.0 (4)
Streptomycin	0.0 (0)	100.0 (6)	0.0 (0)	100.0 (1)	0.0 (0)	100.0 (2)
Amikacin	0.0 (0)	100.0 (8)	0.0 (0)	100.0 (5)	20.0 (1)	80.0 (4)
Kanamycin	0.0 (0)	100.0 (7)	0.0 (0)	100.0 (5)	20.0 (1)	80.0 (4)
Doxycycline	25.0 (2)	75.0 (6)	100.0 (5)	0.0 (0)	60.0 (3)	40.0 (2)
Lincomycin	0.0 (0)	100.0 (8)	0.0 (0)	100.0 (5)	0.0 (0)	100.0 (5)
Azithromycin	12.5 (1)	87.5 (7)	60.0 (3)	40.0 (2)	60.0 (3)	40.0 (2)
Polymyxin B	0.0 (0)	100.0 (8)	60.0 (3)	40.0 (2)	60.0 (3)	40.0 (2)
Trimethoprim	12.5 (1)	87.5 (7)	60.0 (3)	40.0 (2)	60.0 (3)	40.0 (2)

over 60% except for β-lactam group. Some antibiotics that used to effectively deal with Gram-negative bacteria (*H. parasuis*, *P. multocida*, *B. bronchiseptica* and *A. pleuropneumoniae*) such as macrolides and beta-lactams were indicated to be less sensitive, especially lincomycin could not be used for any bacterial isolates. Polymycin B, which is known to use in human treatment, presented 100% resistance by *S. suis* and *A. pleuropneumoniae*, and 40% by *H. parasuis* and *P. multocida*. As a result, only a narrow spectrum of effective antibiotic drugs can be used for the treatment of infection in Hubei pigs.

This study also revealed the number of bacte-



*Note: Beta-lactams (AMOX: amoxicillin, AMP ampicillin, CEFTI: ceftriaxone, CEFRA: cefradine, CEFO: cefotaxime, CEFTA: ceftazidime). Quinolones (OFL: ofloxacin, CIP: ciprofloxacin, ENRO: enrofloxacin, NOR: norfloxacin). Aminoglycosides (SPEC: spectinmycin, GEN: gentamycin, STREP: streptomycin, AMI: amikacin, KANA: kanamycin). Tetracyclines (DOX: doxycycline). Lincosamide (LIN lincomycin). Macrolides (AZI: azithromycin). Polymycin (POLY: polymyxin B). Sulfonamide (TRIME: trimethoprime). SS1 – SS6: *S. suis* isolates number 1 to 6. HPS1 – HPS6: *H. parasuis* isolates number 1 to 5. PM1 – PM5: *P. multocida* isolates number 1 to 5.

Figure 1. The number of bacterial isolates resistant to antimicrobial agents (A) *S. suis* isolates.

rial isolates that exhibited multi-drug resistance (MDR) (Figure 1). According to these data, each isolate of *S. suis* were resistant to at least one antimicrobial drug in more than six antimicrobial categories. Each isolate of *H. parasuis* and *P. multocida* were resistant to at least one antimicrobial drug in two or more antimicrobial categories. The three *B. bronchiseptica* isolates were

also against to at least one antimicrobial agent of beta-lactams, quinolones, aminoglycosides and lincosamides. Similarly, *A. pleuropneumoniae* isolates were resistant to at least one antimicrobial agent of seven tested drug classes, except for macrolides.

The results suggested that five species of bacteria were highly multi-resistant to the eight common drug classes. Multi-drug resistance is a problem that continues to challenge the healthcare sector. Different countries have reported the widespread of clinical resistance due to the massive of antimicrobial drugs (Jong et al. 2018). The transmission of MDR bacteria into the community is seriously associated with increased morbidity, mortality, healthcare costs and antibiotic use. Together with many European countries and the USA, China is preparing a national action to deal with antibiotic resistance. Current technology makes possible the identification of new drugs or inhibitors of resistance mechanisms to extend the life of existing antibiotics, or alternatives like plant extracts (Laxminarayan, 2013). However, these tend to take time and require further efforts. Initial steps to prevent the spreading of MDR is use antibiotics only when needed and correctly, control the usage by reducing antibiotics in livestock management.

Due to different antibiotic usage of different farms, more difficulty and complication have raised in the aspect of antibiotic control of the area. The temporary solution is giving drug regimen based on susceptibility result of individual farms. Long-term plan with a detailed guideline of antibiotic implication should be developed for the control of bacterial disease and protect public health from antimicrobial resistance.

3. Conclusions

The results demonstrated high multi-resistance among the five bacterial species to the eight tested antimicrobial classes. The results emphasize the need for continuous surveillance of resistance patterns. Antibiotic prescription guidelines and infection control through the early detection of clinical should be carried out to prevent transmission of pathogens, as well as in the possible incorporation of the prevalent serotypes in the development of new vaccines.

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Infection status of *Mycoplasma hyopneumoniae* in experimental pigs at a commercial farm

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ABSTRACT

The objective of this study was to investigate the profiles of *Mycoplasma hyopneumoniae* (MH) infection at different ages of pig in a sow – finishing herd using serological and molecular methods. A total of 30 study piglets were born from non-vaccinated sows with MH. They were injected one-dose of inactivated MH vaccine at the 10^{th} week. MH infection status was evaluated by using ELISA to detect MH antibodies from blood samples, and PCR to detect MH DNA in nasal swabs or oral fluid samples every other weeks from newborn to slaughter time. The results of this study showed that PCR positive proportions were low at 1^{st}-2^{nd} week (7-13%), then increased significantly during 5^{th} -7^{th} week (73-79%), and reduced at 8^{th} week (33%); finally became negative after 13^{th} week of age. This pattern corresponds to the one of antibody level. In particular, the level of maternal antibodies against MH was very high due to maternal immunity, then decreased gradually to negative at 7-8 weeks of age, and finally increased gradually from 13 weeks of age to all positive at 25 weeks of age. In conclusion, the result showed that in this herd, MH might invade pigs by the time of 5-7 weeks of age after maternal immunity disappears, and humoral response can overcome the infection at week 13. This should be noted to have appropriate strategies to control MH at the farm.

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1. Introduction

Mycoplasma hyopneumoniae (MH) is a principal aetiological agent of porcine enzootic pneumonia (EP), a respiratory disease that mainly affects growing and finishing pigs (Maes et al., 1996). MH infection causes damage to the lung lesions, and modulates immune response of the host. MH primary infection often becomes more serious when getting co-infections by other bacteria and viruses such as *Pasteurella multocida*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae* (APP), Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), and Porcine Circovirus type 2 (PCV2), etc. leading to a complication called Porcine Respiratory Disease Complex (PRDC) (Thacker et al., 2000). Once infected, pigs become stunted, low growth rate, poor feed conversion ratio (FCR), as a result of high culling rate in the herd, massive cost of treatment, and getting more susceptible to secondary pathogens (Thacker & Minion, 2012). It is estimated that approximately 80% of pig production had been affected with the disease and every one infected pig cost approximately 4-7 USD (Haden et al., 2012).

In order to evaluate the effectiveness of the vaccination plan, it is essential to get a better understanding the situation of MH infection throughout stages of production in farm. The objective

of this study was to investigate the dynamics of MH infection at different ages in a pig herd by using ELISA and PCR to detect both antibodies and the bacterium DNA. The result of this study also helped to estimate the infected time and risk period under field conditions.

2. Materials and Methods

2.1. Experimental design

The study was conducted from February 2019 to October 2019 in a medium – scale pig farm with a scale of 1000 grow-finisher pigs and 200 sows, a type of open-housing system, in Xuan Loc district, Dong Nai Province.

A total of thirty piglets from five 3^{rd}-5^{th} parity sows that these sows had been checked to be free of PRRSV, CSFV and MH based on PCR tests (one week before farrowing) on individual oral fluid samples and determined level of antibody against MH basing on ELISA test (one hour after farrowing) was enrolled in the study. From each sow, 3 male and 3 female newborn piglets with the same size and the same body conditions were selected, and individually marked by ear tags from number 1 to 30, raised stable during the whole period of the study. According to vaccination program of farm, all these piglets were injected one-dose of Bayovac® MycoGuard®-1 vaccine at the 10^{th} day of age. The piglets were weaned at 24 days-old and mixed together in only one pen (basic floor pen) until they were transported to slaughterhouse.

The MH infection status of experimental pigs was determined via testing of both blood samples and nasal swab/ oral fluid samples at different ages. Sampling timeline was designed according to life-stage of study pigs, i.e. the first 60 days of age (week 1-8); nursery phase (week 9-12) and finishing phase (week 13 – 25). In particular, individual blood samples were taken from study pigs based on week-age, i.e. week 1, 2, 4, 5, 7, 8, 13, 19 and 25 weeks, respectively. In addition to blood samples, individual nasal swabs were collected for the first 8 weeks of age, however, pooled oral fluid samples were collected for whole studied group at the later stages (week 13, 19 and 25).

Each sampling time, only 50% of studied pigs would be sampled and 50% remain pigs would be sampled at the next time to avoid piglets having been bled for 2 consecutive weeks. In details, at the 1^{st} week, 3 piglets per litter were selected alternately male or female to collect samples for every 2 weeks, and at the following week the other half would be sampled for every 2 weeks. It means a total of 15 piglets were assigned to take samples per week throughout the timeline except for the week of weaning.

2.2. ELISA and PCR procedures

From the nasal swabs and oral fluids, DNA was extracted to run a standard PCR to detect a fragment of 16S rRNA gene of MH. The assay was previously described and performed by using primers according to Abhijit et al. (2012) with the specific primers (sequence with 5' – 3' direction) for DNA amplification (F: ACTAGATAGGAAATGCTCTAG and R: ATACTACTCAGGCGGATCATTTAAC) to have a product of 430bp in length. Blood samples were stored in cool condition for less than 24 hours, after that serum was aspirated from the tube and frozen in refrigerator -20°C until analysis. These serum samples were analyzed for the presence of antibodies against MH with an indirect ELISA (IDEXX *M. hyo.* Ab test kit, USA). The output of ELISA was read with a 650 nm filter to calculate the S/P value of each sample. The result is defined as positive when S/P ratios were > 0.4, S/P ratios of 0.3 to 0.4 were classified as suspect and S/P ratios < 0.3 were classified as negative. MH antibody titer was evaluated from S/P using the formula recommended by the kit producer: Titer = $\text{Antilog}_{10}(1.09 * \text{Log}_{10}(\text{S/P}) + 3.36)$. These laboratory procedures were performed at the diagnostic center of Veterinary Hospital of Nong Lam University, Ho Chi Minh City, Vietnam.

2.3. Statistical analysis

Data was managed and performed simple analysis using Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Proportions of sample number being positive were calculated, and means of titer with standard error were calculated for each sampling time. Multilevel regression was used to model the pattern of MH titer in which dependent variable was titer, independent variables included week age, quadratic week age, cubic week age, sex (male/female), day-0 weight, maternal MH (positive/negative), and litter identification was random variable. Backward elimination ap-

proach was used to build the final model with the statistical significance level (P) of 0.05. The final parameter model results are applied to a simulation data for graphing dynamics of MH infection of pig in the herd. These steps were performed with STATA 14 software (StataCorp., 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

3. Results and Discussion

3.1. Detection of MH by PCR

The presence of MH detected by PCR in nasal swabs (week 1 – week 8) and oral fluids (week 13-week 25) are shown in Figure 1.The MH infection proportion at the first week was 7% (1/15), then it gradually rises to 13% and 20% at week 2 and week 4, respectively (Figure 1). A significant increase of MH infection is observed and reached 79% at week 7. However, at 8 weeks of age, the MH infection proportion dropped markedly to 33%.

After 8 weeks of age, the number of samples required to detect MH DNA of study pigs is high, which result in costly diagnosis. To overcome some of these limitations, instead of taking individual nasal swab samples, we obtained pooled oral fluid samples for the group of study pigs to perform PCR. For the pooled oral fluid samples, all of them were negative for MH at week 13, week 19 and week 25. It was generally interpreted that the individual could also be considered all study pigs were negative with MH or MH infection rate was in very low level, so that the result was negative at all.

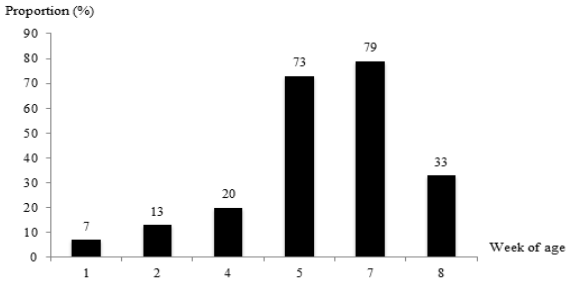

Figure 1. The MH infection proportion defined by PCR in pigs by the week of age.

MH infection at week 1 was the lowest could be explained by negative MH shedding sows selected and the effects of the passive transfer of maternal MH antibodies and specific cellular immunity to piglets via colostrum. The maternal immunity are known critical to prevent or reduce the impact of infectious diseases in the neonate for a few days to several weeks after birth. In the studied farm, MH vaccination is applied for piglets not in sow. That means enzootic pneumonia might be endemic in a sow herd particularly in continuous production systems (Sheldrake et al., 1990; Bandrick et al., 2008), and the maternal immunity are ready in sow in such level to transfer to piglets. In fact, all sows were negative in PCR result for MH but 3/5 sows were positive with antibody by ELISA (data not shown). And MH might be from the environment to accidentally infect to a pig.

From week 2, maternal MH antibodies have not been enough to help them fight the disease; however, these suckling piglets are in nursing phase so that rarely exposed to the external environment, the proportion was increasing slowly. The weaning age of 21 days was the time that the maternally immunity eventually wanes (Meyns et al., 2004). These piglets separated from their sows experienced marked physiological, environmental, mixing and social challenges (stressors) that could predispose them to MH infection. Therefore, the period between week 4 and week 7, it was the potential to increase the susceptibility of piglets to get infection by impact of MH in the environment and from the other infectious penmates.

The infection proportion began to diminish and especially reach zero with MH at week 13, 19 and 25 by pooled oral fluid samples. It was generally supposed that the results of these pooled samples could be as follows: if the results are negative, the individual could also be considered all study pigs were negative with MH or MH infection rate was in very low level inconsiderably, so that the result was negative at all. It is known that the high-risk period of MH infections occurrence under field production conditions is the phase after transfer of animals to the finishing facilities (10 weeks of age) (Léon et al., 2001). Moreover, during this period, the farm increased the use of antimicrobials, minerals and vitamins via feed and water to control MH and maintain pig health. Thus, these antimicrobials for the treatment and control of MH infections could be helpful in affected pigs. Based on above considerations, the negative results of pooled oral fluid samples at every sampling time demonstrated for efficiency of antibiotics on reducing the positive rate with

MH infection by PCR. These findings is similar to the previous study that all pen-based oral fluid samples for MH in finishing phase were negative (Sibila et al., 2007). Piglets were vaccinated with inactivated vaccine which might slow induce immunity, but at these points of time, high level of antibody from field infection and vaccine could boost to the level of eliminating the bacteria. Finally, these results indicated the presence of MH in the respiratory tract, which could be related to the presence of antibodies in the blood of study pigs.

3.2. Detection of MH antibody

After performing ELISA tests for serum samples, the MH antibody positive proportion and means of titers by week age are illustrated in Figure 2. At the first week of age, the antibody positive proportion with MH was highest (53%), equivalent to the highest antibody titer of 1532.47. After that, the rate of positive serum for MH began to decrease from the second week (proportion of 40%, titer of 904.04) to week 8, only 0%, equivalent to the antibody concentration of 204.59. Then, the antibody positive proportion as well as mean of titer increased significantly and reached 100% (1321.59) at week 25. These results coincide with those obtained in field studies using ELISA by Morrison et al. (1985) who noted that antibodies to *M. hyopneumoniae* were detected again at 90 to 150 days of age, and Sheldrake et al. (1990) reported that most pigs seroconverted between 86 and 144 days of age.

According to Figure 1 and Figure 2, the MH infection status was illustrated compatibly when positive ratios in PCR and ELISA result had contrary directions. In the present study, high prevalence of MH infection occurred around the time of post-weaning period until beginning of finishing period. The critical moment for the exposure to *M. hyopneumoniae* was around 9-10 weeks of age and most of them have very low concentrations of antibodies against the agent.

3.3. Modeling of antibody titer against MH

Antibody titer values from the studied piglets by age were modeled to understand the pattern of its change and any other related factors such as gender, body weight, maternal antibody, etc. The result from modeling found that week-age has a cubic relationship with antibody titer. Maternal antibody (MAB) in this model is a binary variable in which the sow transferred MH antibody to piglets or not. The reason is that each piglet can receive different level of MAB. The other concerned variables were not significant in the modeling construction. The final model is described in Table 1 and the simulation of this model can be seen in Figure 3. The positive result was confirmed when S/P ratios were > 0.4, so the cut-off value was calculated as 843 according to the kit formula with $S/P = 0.4$ to classify boundary of MH titer with or without MAB.

According to modeling illustration, we found that the average age at which piglets lost protection lies well between 2^{nd} week and 4^{th} week. The titer of pigs having MAB did not decline as rapidly as those of without-MAB pigs. Additionally, we observed that the lowest level of antibodies was in the period from 8th week to 10th week of age; and protection afforded by MAB had higher level than piglets lacking of MAB. Afterwards, from week 16 to 20, the diagram indicated that both groups had a seroconversion that the antibody level reached to detectable values and continued to increase. However, we assessed that MAB group increased titer earlier than that of the without MAB pigs.

3.4. General discussion

Thacker et al. (2000) suggested that both local mucosal antibodies and systemic cell-mediated immunity responses are important for protection. Therefore, by using serum to detect IgG antibodies to MH by ELISA, this study cannot evaluate the mucosal antibody because MH is a mucosal pathogen which mainly adheres to the cilia of the epithelial cells on the respiratory tract, the production of IgA antibody blocking MH attachment to the mucosal surface is believed to play a key role in protection (Zhang et al., 1995). It is generally that IgA predominates in the mucosal secretions, whereas IgG predominates in serum. However, there was no correlation between antibody titer or IgG concentrations in serum and level of protection against MH infection (Djordjevic et al., 1997). Thus, it is difficult to link the antibody to the presence of MH on the respiratory track, and this presence cannot refer to infection. However, at least, the antibody level in serum can imply the time of infection in piglet. That means it is valuable comparing to PCR which might more refer to the high risk time.

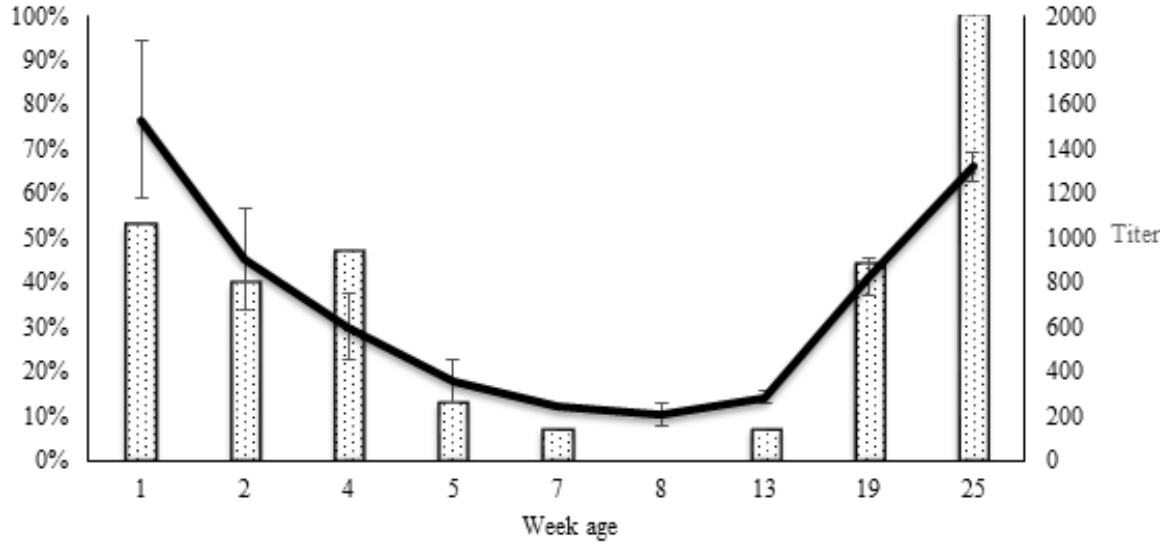

Figure 2. Antibody positive percentage (bars) and antibody level against MH ± SE (line) in pigs by week of age.

Table 1. Modeling of piglet antibody titer values by variables

Variables	Coefficient	95% Confidence	Interval	P value
(Week age)	-391.825	-499.498	-284.152	< 0.001
$(\text{Week age})^2$	28.678	18.921	38.435	< 0.001
$(\text{Week age})^3$	-0.549	-0.794	-0.305	< 0.001
MAB	383.192	213.323	553.062	< 0.001
Constant	1510.820	1193.526	1828.114	< 0.001

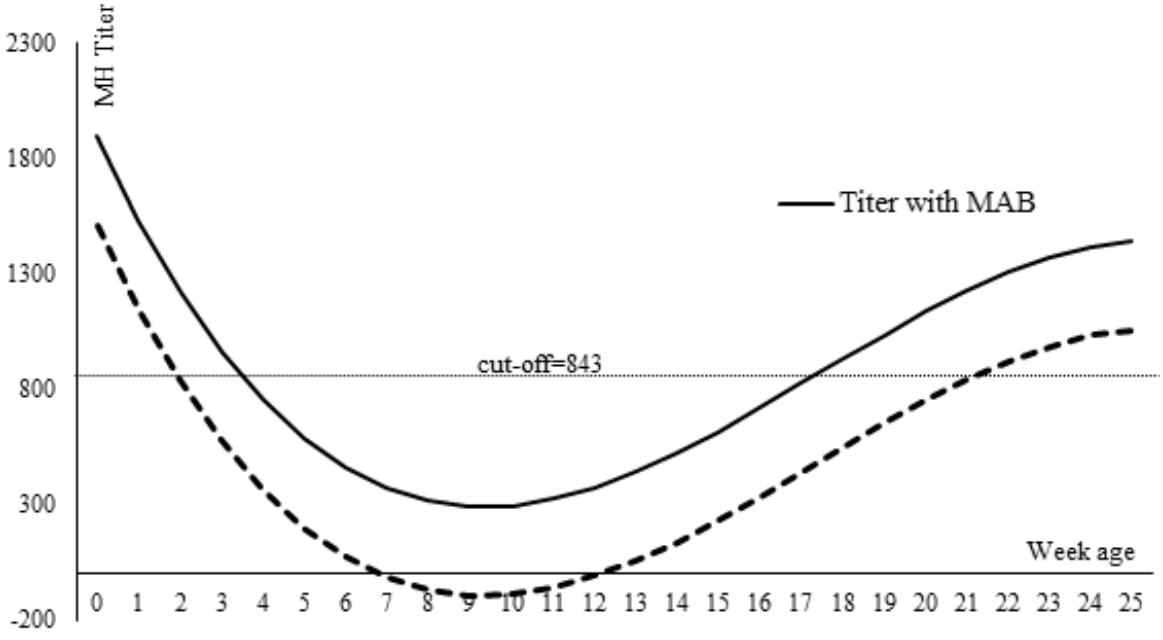

Figure 3. Modeling pig MH antibody titer values by variables (week age, with or without maternal immunity).

Resistance to the disease after recovering appears to be dependent on a balance between the immune status of the animals and the pathogen load. In the study, those pigs received antibiotics like other herds in farm via feed and water additives. Additionally, under field conditions, antimicrobial treatment may be effective against bacteria respiratory pathogens specifically MH, and it can be implemented to reach a low infectious pressure in the farm at that moment (Thacker & Minion, 2012). This given medication modifies the pig' microbiota and alteration of epithelial mucosal bacteria influences development on the study pigs' respiratory immune system (Arsenakis et al., 2017). Thus, besides vaccination, several treatment strategies should be considered as the sole to mitigate expression of disease and reduce prevalence within herd.

4. Conclusions

High prevalence of MH in the farm and the infection occurred from the time of 2-3 weeks after weaning until beginning of finishing period, weeks 9-10.

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Case report of brachycephalic obstructive airway syndrome in brachycephalic dogs from Veterinary Specialist Service Hospital, Australia

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ABSTRACT

This report aimed to study symptoms and causes of brachycephalic obstructive airway syndrome (BOAS) in brachycephalic dogs and to determine appropriate surgical procedures for these symptoms by reviewing literatures and examining four case studies conducted at Veterinary Specialist Service Hospital, Underwood, Queensland, Australia. The cases included a 6-year 3-month old Staffordshire Bull Terrier (case 1), a 1-year 5-month old French Bulldog (case 2), an 8-month old French Bulldog (case 3), and an 8-year 8-month Pug (case 4). Those dogs went to the Veterinary Specialist Service in a worsen state of respiratory problems, including the upper respiratory noise (case 1, 2, 3), decrease in exercise tolerance, respiratory struggling (case 1, 3), regurgitation (case 1), coughing, sleeping difficulty, respiratory stridor (case 2), nasal discharge, dyspnea, bloating, and tachypnea (case 4). Examinations revealed the causes including the elongated soft palate (case 1, 2, 3, 4), stenotic nostrils (case 2, 3, 4), tonsils inflammation (case 3) and everted laryngeal saccules (case 4). After surgery, the dogs were recovered in intensive care unit within 2 days, and then discharged. Scheduled re-examination one week later showed improvement in the respiratory health in all cases. Overall, major complications occur in 10% of cases; however, this surgery is vital and can be totally applied in Vietnam where brachycephalic dogs have become a popular companion.

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1. Introduction

Congenitally, brachycephalic dogs are characterized with a shortened head, which is brachycephalic syndrome also known as brachycephalic conformation of the skull (Bjorling et al., 2000). The syndrome consists of anatomic abnormalities including stenotic nares, tortuous turbinates, caudally displaced maxillae, elongated soft palate, everted laryngeal saccules, and hypoplastic trachea (Ackerman, 1999; Koch et al., 2003). The abnormal skull's anatomy narrows the lumen of upper respiratory tract, thus lead to asphyxiation and collapse during excitement, hot weather or exercises (Koch et al., 2003; Packer et al., 2012). Moreover, the displaced maxillae and the elongated soft palate interfere with laryngeal functions resulting in respiratory stridor, open-mouth breathing, inspiratory dyspnea, exercise intolerance, noisy breathing, suffocating and coughing (Ackerman, 1999; Dupre, 2008). According to skull measurements, the typical brachycephalic breeds include Chihuahua, Bulldog, King Charles Spaniel, Pug, Boston Terrier, Maltese, Pekingese, Miniature Pinscher, Shih Tzu, Yorkshire Terrier, and Boxer,

Lhasa Apso, Shar Pei (Koch et al., 2003). In recent years, the brachycephalic dogs have become the popular breeds in many countries as well as in Vietnam, which inevitably leads to an increase in BOAS cases (Best et al., 2016).

Treatment-wise, hypoplastic trachea, foreshortened maxillae, and narrow rima glottidis are unchangeable. For the case of tortuous turbinate, the surgery can remove a little piece in turbinate to make the airway more ventilated but it is dangerous and expensive with long surgical time. However, the surgery can also affect the patient's olfactory ability, therefore, it is often deemed unnecessary. For the remaining anomalies, there are safer procedures to relieve the symptoms of BOAS, which include trimming the stenotic nares, resecting the elongated soft palate, removal of the everted laryngeal saccules, and removal of the tonsils (depending on the specific situation).

In general, the surgery of stenotic nares includes nares amputation, wedge resection (alarplasty) and alapexy (Fossum, 2013). Specifically, in wedge resection, stenotic nares are resected easily by cutting the V-shaped section of the nares with the No.11 scalpel blade. Wedge resection is less surgical time than alapexy, less incisional bleeding than amputation. However, this procedure can be failed if flaccidity of the cartilage occurs, mobility of the dorsolateral cartilage increases, depigmentation or asymmetrical nose presents. Resection of elongated soft palate is normally performed using Metzenbaum scissor. Electrosurgery can also be used instead; however, may cause swelling in post-operative care. In some cases that tonsils inflamed or obstruct the airway, tonsils can be removed by Metzenbaum scissor or scalpel blade. Then, at the base of the everted tissue, using the tip of a long-handled, curved Metzenbaum scissors transects the everted laryngeal saccules.

To determine which surgical procedures are suitable for the patients, clinical examination and diagnosis are conducted. In clinical examination, the stenotic nares, the size of the trachea and the obstructive inspiratory dyspnea with stertor can be determined by observation and palpation. After that, diagnostic radiography and bronchoscopy rule out abnormal respiratory and cardiology diseases. The surgical procedure should be performed as soon as possible for dog that is above 4 months of age since the nasal tissues are mature enough to hold sutures.

2. Materials and Methods

2.1. Case 1

2.1.1. History

Dog 1 was a six-year-three-month-old male neutered Staffordshire bull terrier breed dog. Noisy breathing was observed by owner; however it became worse during the past year, together with exercise intolerance, heat intolerance, and respiratory struggle during excitement. The dog would occasionally regurgitate white foam, despite eating and drinking normally without coughing or sneezing, or changing in bark.

2.1.2. Clinical examination

Clinical examination revealed lean body condition, noticeable upper respiratory noise, pink mucous membrane, good airflow through both nostrils; elongated soft palate was observed. Based on the result, the dog was diagnosed with signs of BOAS. The obstruction of airway was likely due to elongated soft palate and small probability of laryngeal paralysis, with recommendation for BOAS surgery and arytenoid lateralization.

2.1.3. Laboratory test

The result of the blood test was 37% and 70 for packed cell volume and total protein respectively, which is ordinary according to the normal range of PCV (37-55%) and normal range of TP (55-75). This test is quick, easy and it is a common preoperative test because it gives information of the patient's status about anemia, blood protein, hydration status.

2.1.4. Radiograph

Findings in thoracic radiographs were unremarkable (Figure 1). The result of imaging diagnosis showed that there were no cardiology and respiratory problems such as heart base tumor, nasopharyngeal, laryngeal, tracheal masses; and secondary changes to the lungs, such as bronchiectasis and probable hypoplastic trachea.

2.1.5. Bronchoscopy

The upper airway was examined with bronchoscopy showing the arytenoids moved bilater-

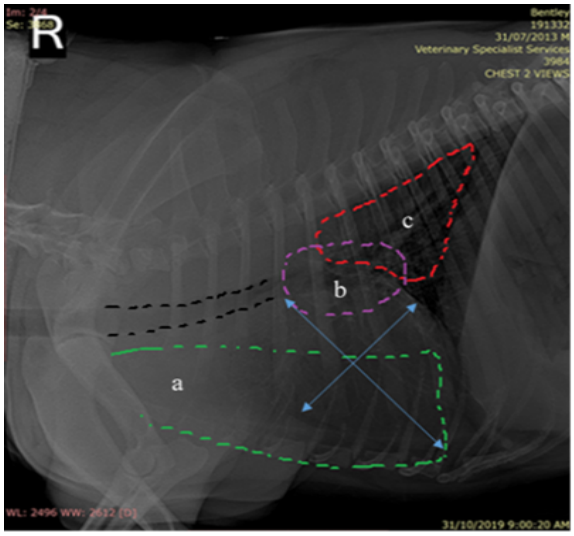

Figure 1. The right lateral thorax radiography (Dog 1). Ventral side (a), Perihilar (b), Dorsocaudal (c) and diffuse of lungs assessed for bronchopneumonia, especially aspiration pneumonia, pulmonary edeama, pulmonary haemorrhage. Vertebral heart size (VHS)= 8 (< 10.7), heart size was normal. Tracheal hypoplasia was rejected (black line) and no hiatus hernia, no abnormalities in vertebral body.

ally, hence arytenoid lateralization was deemed unnecessary.

2.1.6. Surgery

The surgical procedure included general anesthesia, upper airway exam, thoracic radiographs and elongated soft palate resection. The patient was then pre-oxygenated for 5-10 minutes and slowly induced with Alfaxalone 30mg IV, and intubated with a cuffed ET tube. Circulation, heart rate, oxygenation, ventilation, blood pressure values including systolic arterial pressure, diastolic arterial pressure, mean arterial pressure and the anesthetic maintenance was updated every five minutes using intraoperative monitoring system.

First, the patient was placed in sternal recumbency with the mouth fully opened and the chin was not allowed to have contact with the table's surface. Next, the mucosal surfaces should not be scrubbed to protect from irritation and edema; the endotracheal tube was secured to the lower jaw ensuring free access to the soft palate. Then, the surgeon began to scrub and prepared the soft palate kit when everything was in position (Figure 2).

1. Debakeys: 1X-long, 1 Normal
2. Rat tooth Forceps Long
3. Adson Forceps
4. No.7 Scalpel Handle
5. Suture Scissors
6. Metzenbaum's: 1x Short, 1x x-Long
7. Laheys x 2 Short
8. Needle Drivers – 1 Short, 1 X-Long
9. Artery Forceps: 3 x Small, 1 x Large
10. Laheys x 1 Long
11. Allis Tissue Forceps x 1
12. Swabs
13. Kidney Dish
14. Hand Towels

Figure 2. List of surgical instruments for BOAS surgery.

For elongated soft palate resection, the resection was done with scissors, the surgeon placed stay suture of 3-0 Monocryl at the proposed site of resection. Next, the surgeon resected one third of the soft palate with Metzenbaum scissor, then apposed the mucosa with 3-0 Monocryl simple continuous suture pattern; the procedure was then repeatedly continued between excision and suturing until the resection was completed (Figure 3). During the resection, there was mild hemorrhage, which was put under control by tying a swab to a thread and placed the swab in the surgical area.

2.1.7. Post-operative care

After surgery, the patient was moved to the Pet intensive care unit (PICU) to recover overnight with close monitoring. Postoperative care included the late extubation, analgesic protocol, nasal oxygen supplementation, close monitoring of the breathing. Upper airway obstruction in post-surgery was concerned due to inflammation and swelling. After surgery, Medetomidine was needed for anxiety, with transition to Tramadol Oral the next day. Post-surgery medications: Methadone 0.1 - 0.2 mg/kg SC and Medetomidine CRI at 1 µg/kg/h. After 2 days in the PICU, the dog swallowed the food trial and was discharged.

2.2. Case 2

2.2.1. History

A one-year five-month-old female French bulldog was examined for a history of some upper respiratory noise and coughing. The dog ate quickly and sometimes slightly choked on food. Otherwhile, the dog had not shown any significant respiratory difficulty.

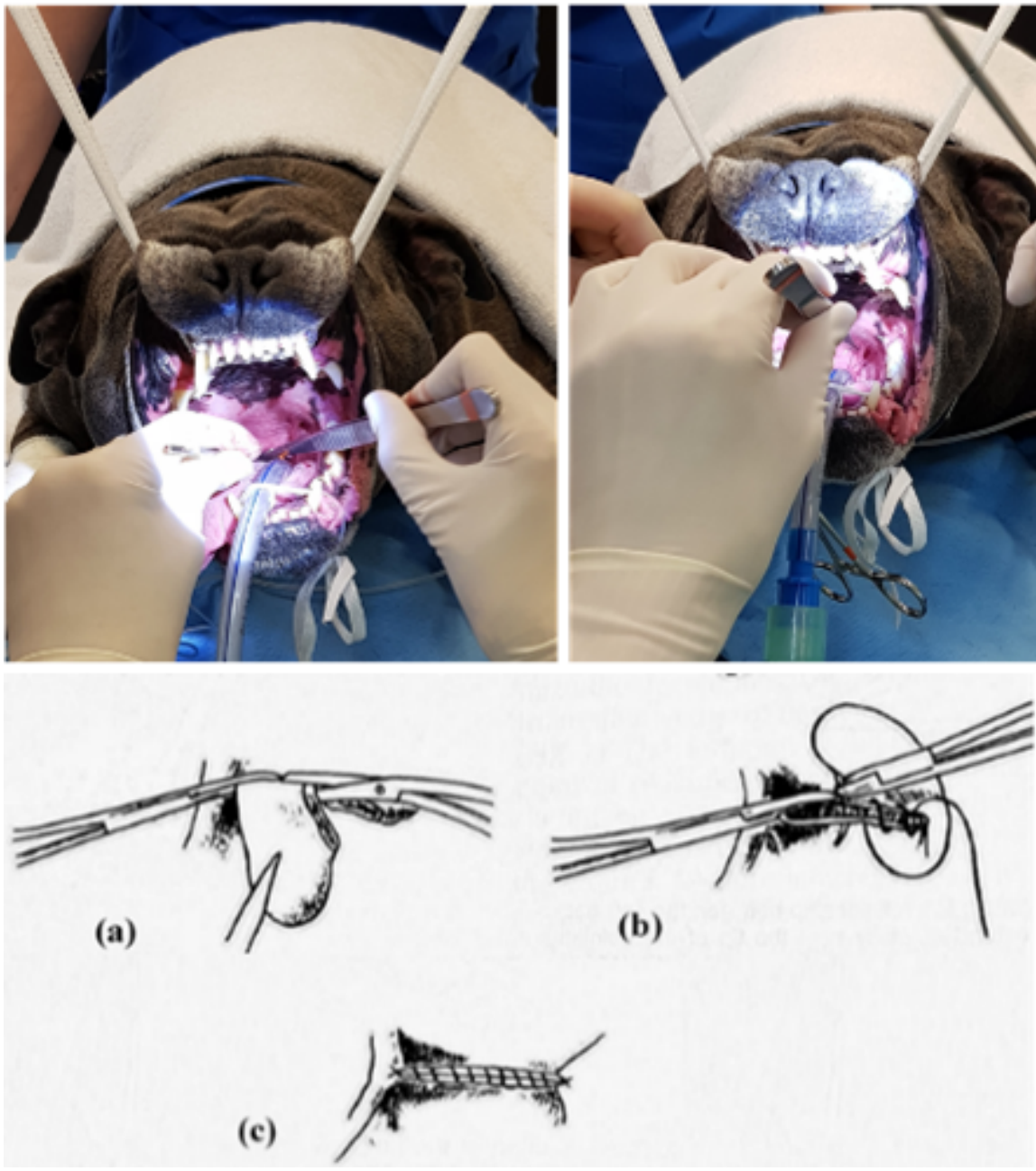

Figure 3. Elongated soft plate resection (Dog 1). (a) Transected one-third of the palate, then (b) apposed the mucosa with sutures. (c) Continue alternating excision and suturing until the resection was completed (Bjorling et al., 2000).

2.2.2. Clinical examination

On initial physical examination, the dog possessed congenital traits of brachycephalic breeds. Due to observational heavy open-mouth breathing, respiratory stridor, and difficulty sleeping, elongated soft palate was considered as a main cause. The dog nostrils were congenital stenotic. Thoracic auscultation showed normal cardiac and bronchovesicular sounds, however there was a slight upper airway noise. Based on clinical examination, the first diagnosis was BOAS and surgery therapy was recommended.

2.2.3. Laboratory test

The result of PCV/TP was normal at 45/74 based on normal range.

2.2.4. Radiograph

The radiograph was conducted to detect other diseases; especially aspiration pneumonia because of the dog's eating routine. The findings were unremarkable (Figure 4).

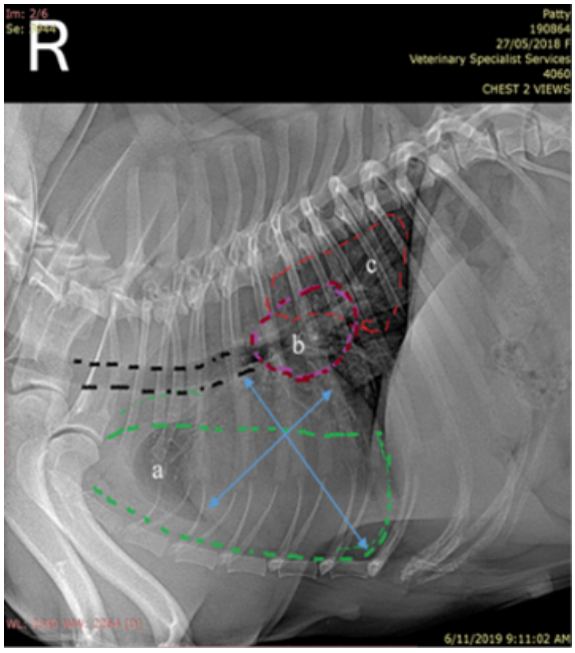

Figure 4. The right lateral thorax radiography (Dog 2). No hiatus hernia, no abnormalities in vertebra, lungs, trachea. Heart size was normal (VHS = 9 < 10,7).

2.2.5. Bronchoscopy

During bronchoscopy, laryngeal paralysis was ruled out; no laryngeal saccules were observed. Main bronchus and secondary bronchi were observed no abnormalities (Figure 5).

2.2.6. Surgery

The treatment regimen included the resection of elongated soft palate and the resection of stenotic nares.

During soft palate resection surgery, a stay suture of 4-0 Monosyn was placed in the caudal midpoint of the soft palate, allowing it to be pulled rostrally. Two additional stay sutures were placed to either side of the soft palate to mark the intended line of resection, level with the caudal tonsillar crypts. The left side of the soft palate was cut with Metzenbaum scissors, then 4-0 Monosyn simple continuous pattern was used to appose the nasal and oral mucosal cut edges of the soft palate. The process was repeated to remove the remaining soft palate. Next, for stenotic nares resection, wedge resection was performed to permanently enlarge the external nares. Using an 11 scalpel blade, a triangular wedge of tissue was removed from the lateral aspect of the nares. Closure was achieved with 4-0 Monocryl, absorbable sutures placed in a simple interrupted pattern (Figure 6).

2.2.7. Post-operative care

After surgery, dog 2 was moved to the PICU for recovery. IV Hartmann's was maintained, meloxicam 1.5 mg/mL was used once per day when eating to reduce postoperative inflammation and pain; cephalothin 1 g/mL IV was supplied. Day 2 in PICU, the patient was stable and swallowed the food trial. The patient was discharged and went back home with administered meloxicam 1.5 mg/mL once per day.

2.3. Case 3

2.3.1. History

An eight-month-old male neutered French bulldog was examined for a history of upper respiratory noise with decreased exercise tolerance. Similar to case 1, the dog struggled on hot days and during excitement. The main presenting problem was mild upper respiratory stertor.

2.3.2. Clinical examination

The dog possessed anatomic abnormality of brachycephalic dog breeds, false positioning of the teeth, open-mouth breathing. Thoracic auscultation showed normal cardiac and bronchovesicular sounds. Based on clinical examination, the dog was diagnosed with BOAS.

2.3.3. Laboratory test

The result of PCV/TP was normal at 33/70 based on normal range.

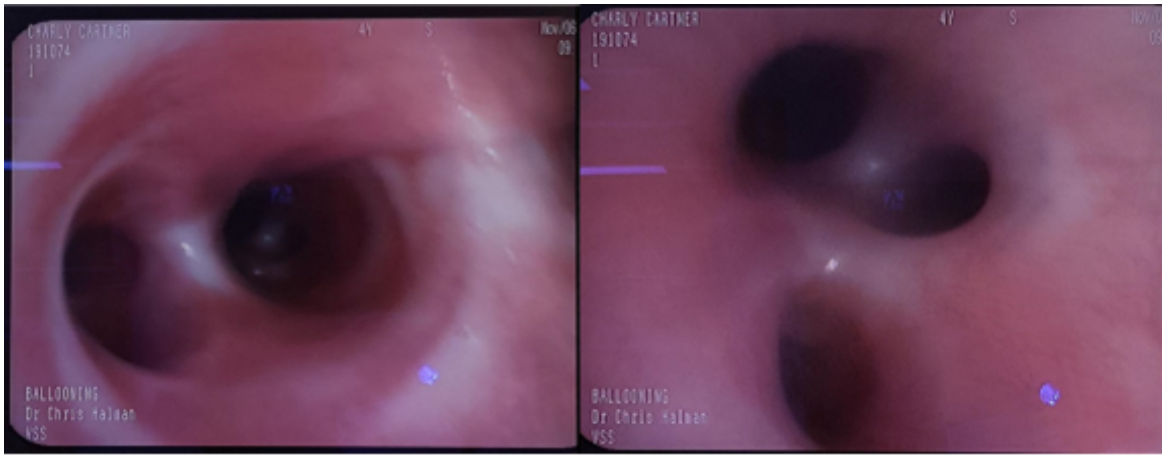

Figure 5. Bronchoscopy (Dog 2). Images of (a) main bronchus and (b) secondary bronchi showed no abnormalities.

2.3.4. Radiograph

Imaging diagnosis showed no other problems (Figure 7).

2.3.5. Bronchoscopy

Oral examination showed the inflammation of tonsils, moderate elongation of soft palate and bronchoscopy showed erythema around arytenoids; there was no evidence of laryngeal saccule eversion or laryngeal collapse, laryngeal paralysis was ruled out.

2.3.6. Surgery

According to diagnosis, the surgeon decided on resection of stenotic nares, resection of elongated soft palate, and tonsils removal. During resection of elongated soft palate, the tip of the soft palate was grasped with allis tissue forceps and a stay suture placed at the site of resection for manipulation. The soft palate was resected approximately one third of the width of the soft palate with Metzenbaum scissors. Closure achieved using 4-0 Monosyn in a simple continuous suture pattern. During resection of stenotic nares, the margin of the nares was grasped with forceps. A V shaped incision was made medially and the second incision laterally. The wedge of tissue was removed and haemorrhage controlled with pressure. The ventral margin of the nares and the mucocutaneous junction were aligned and sutured closed using 4-0 Monosyn in a simple continuous pattern. For Dog 3, the airway was too small, the tonsils were tonsillitis, obstructed the airway. The surgeon proceeded to remove the protruded tonsils (Figure 8).

2.3.7. Post-operative care

After surgery, the dog was moved to the PICU. The dog was noted allergic to Cephalothin, recovered well from anesthesia and surgery, had one regurgitation but was comfortable. The dog continued to be hospitalized in ICU overnight for close monitoring post brachycephalic general anaesthesia and was monitored for risk of aspiration pneumonia. Day 2 in PICU, the dog swallowed food trial well, was bright and alert. Dog 3 showed no regurgitation or emesis, but did have an episode of hypersalivation which was responsive to Maropitant administration which was used the previous night. The patient went back home with Meloxicam 0.1 mg/kg PO SID when eating.

2.4. Case 4

2.4.1. History

Dog 4 was an eight-year eight-month-old female Pug with a history of hemivertebrae and ataxia problems. The dog had a surgery to place plate and decompress at two-year old. Based on the hospital transfer record of Dog 4, carprofen injection was used for reducing spinal pain. In the present time, the dog had nasal discharge, dyspnea and bloating; these episodes gradually became more serious and worsened.

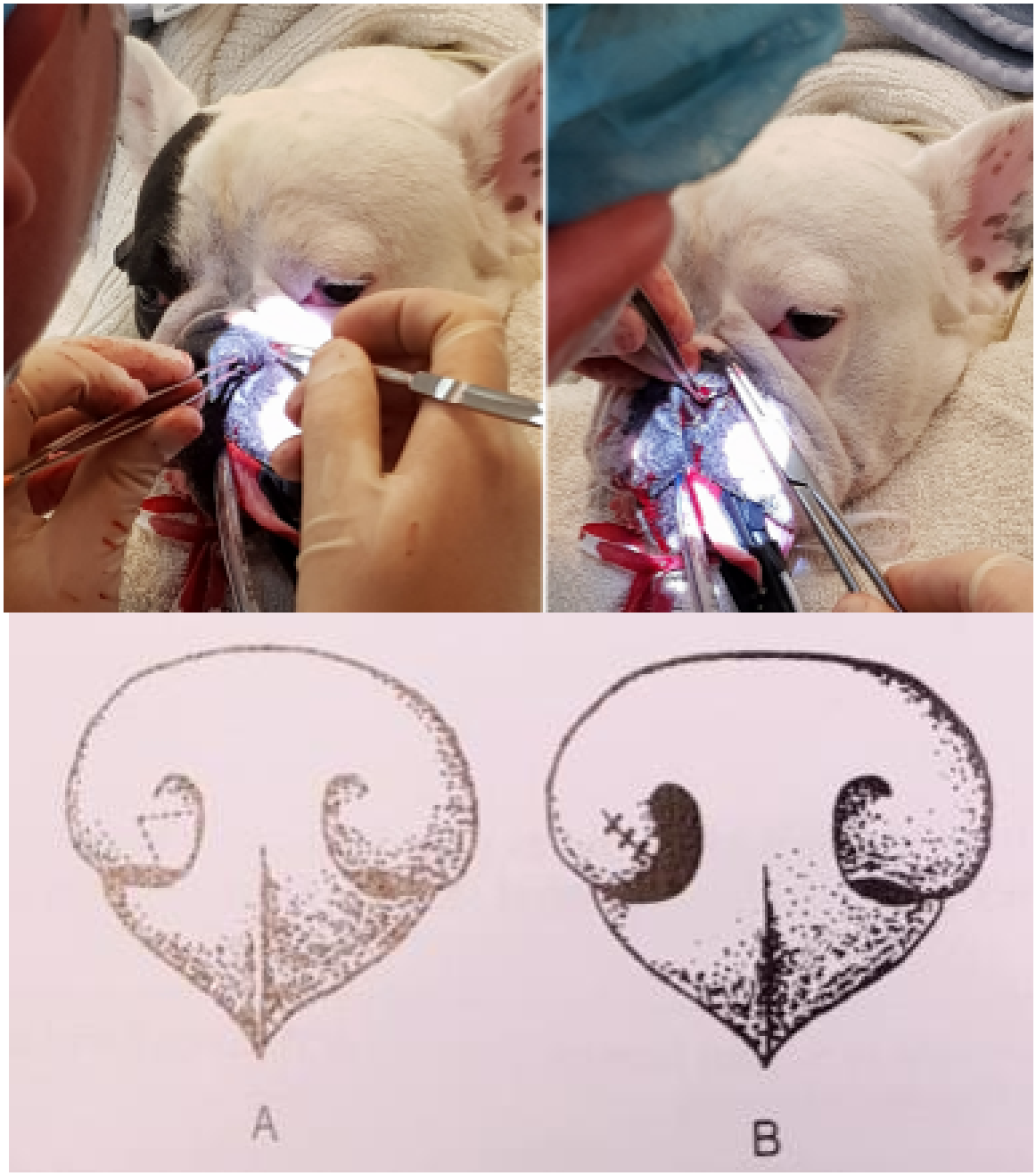

Figure 6. Trimming of stenotic nares (Wedge resection) (Dog 2). (A) Made a V-shaped incision around the forceps with a No. 11 scalpel blade, the first incision was located medially and the second incision laterally. (B) Closed incisions with 4-0 Monocryl, absorbable sutures placed in a simple interrupted pattern (Bjorling et al., 2000).

2.4.2. Clinical examination

Tachypnea and moderate respiratory effort were observed. The nares were narrowed, although not severely. Upper respiratory tract noise was increased.

The dog was examined in four days later for assessment of BOAS following an episode of acute dyspnea and bloating before. At this point, clinical signs were consistent with secondary airway obstruction to BOAS. The obstruction was likely associated with elongated soft palate, stenotic

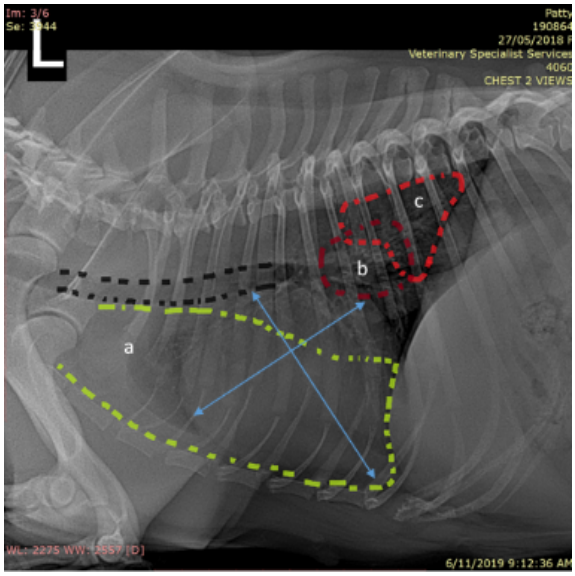

Figure 7. The left lateral thorax radiography (Dog 3). Heart size was normal (VHS = 9 < 10,7). No hiatus hernia, no abnormalities in lungs, trachea.

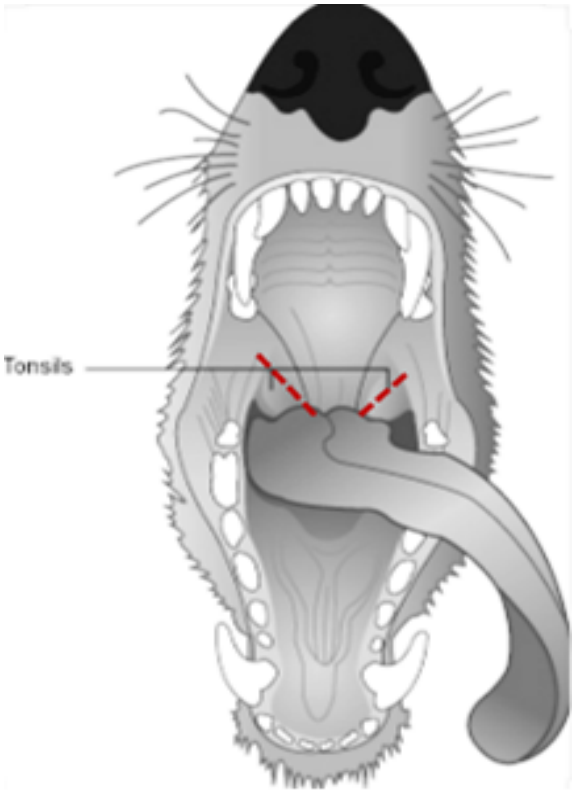

Figure 8. Tonsillectomy (Red lines). Tonsils are removed by forceps and Metzenbaum scissor (Ward & Hunter, 2009).

nares and secondary airway abnormalities. Furthermore, the bloating and associated dyspnea noted might also be in part due to gastrointestinal causes, such as a hiatal hernia. Other causes of airway obstruction or respiratory distress could not be ruled out but were investigated prior to surgery.

2.4.3. Laboratory test

The PCV/TP test result was normal at 40/70. The ALT in biochemical blood test was higher than normal and this was a caution to use medicine in its treatment (Table 1).

Table 1. The result of biochemistry test (Dog 4)

Test	Result	Unit	Lowest value	Highest value
HEM	29.0			
LIP	109.0			
ICT	0.0			
ALB	37.0	g/L	25.0	44.0
ALP	33.0	U/L	20.0	150.0
ALT	156*	U/L	10.0	118.0
AMY	464.0	U/L	200.0	1200.0
TBIL	4.0	µmol/L	2.0	10.0
BUN	6.1	mmol/L	2.5	8.9
CA	2.64	mmol/L	2.15	2.95
PHOS	1.27	mmol/L	0.94	2.13
CRE	82.0	mmol/L	27.0	124.0
GLU	5.1	mmol/L	3.3	6.1
NA^+	147.0	mmol/L	138.0	160.0
K^+	4.6	mmol/L	3.7	5.8
TP	74.0	g/L	54.0	82.0
GLOB	37.0	g/L	23.0	52.0

2.4.4. Radiograph

Radiographs revealed some evidence of periimplant lucency which was suggestive of loss of implant stability, but surgical site comfort and palpation was unremarkable (Figure 9). Lung fields were normal with no evidence of aspiration pneumonia.

2.4.5. Bronchoscopy

Evaluation of the pharynx showed a small central white nodule; different diagnosis was likely an inflammatory nodule.

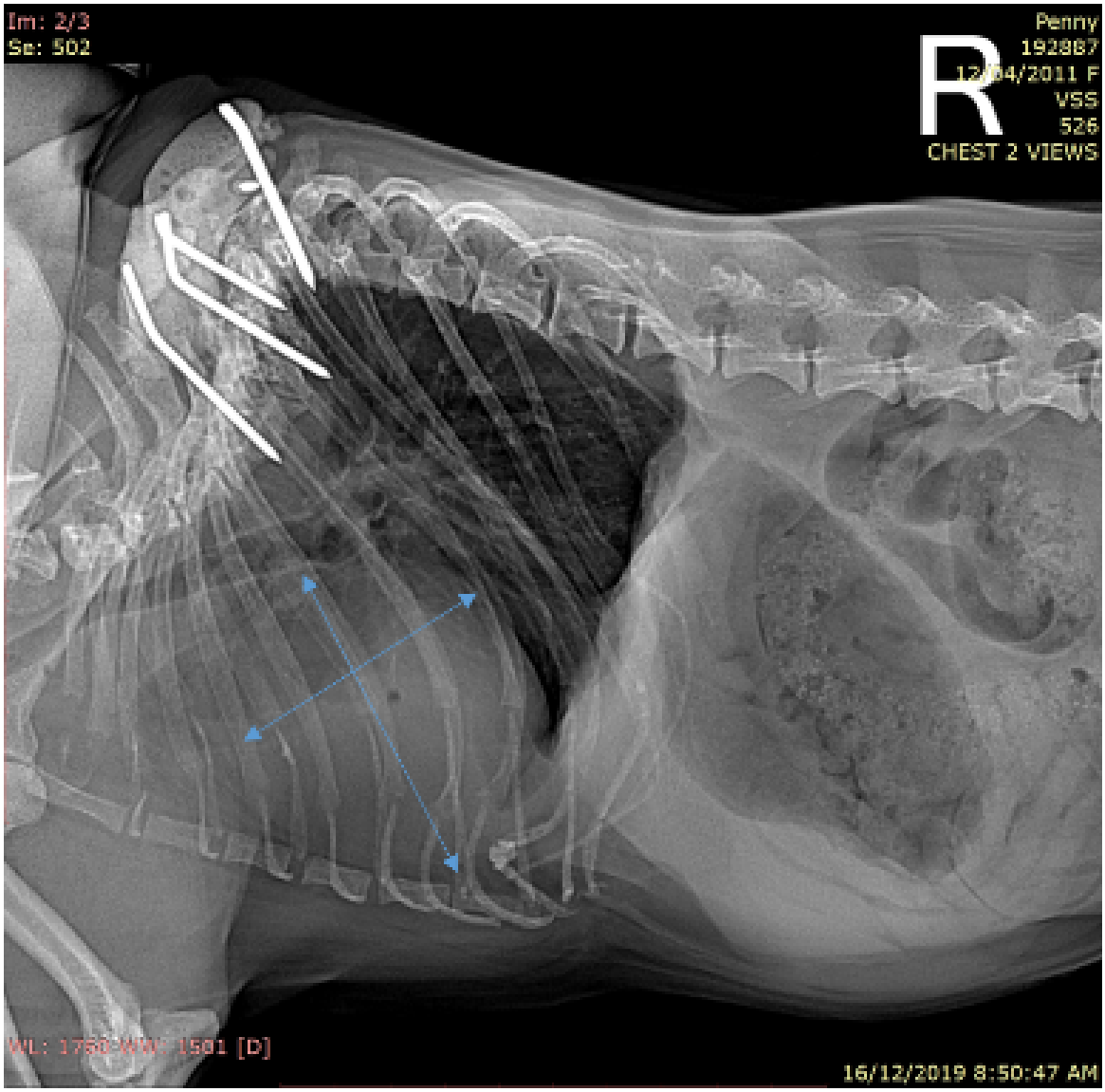

Figure 9. The right lateral thorax radiography (Dog 4). Radiography showed no abnormalities in trachea, lungs, heart, no hiatus hernia. Based on the hospital transfer record, the dog had surgical treatment of hemivertebrae.

2.4.6. Surgery

Surgery was recommended for the dog to reduce resistance to inspiration by conducting a combination of alarplasty, soft palate resection and laryngeal sacculectomy if required. First, in staphylectomy, right angle forceps were used for positioning the soft palate and resected using Metzenbaum scissors. Closure achieved using 4-0 Monocryl simple continuous suture pattern. Second, in removal of the laryngeal saccules, the surgeon temporarily extubated Dog 4. The everted laryngeal saccules were cut off with Metzenbaum scissors (Figure 10). Last, trimming of stenotic nares was performed. A wedge resection of the nares was removed using an 11 scalpel blade. Closure was achieved using 4-0 Monocryl simple interrupted suture pattern.

2.4.7. Post-operative care

After surgery, the dog recovered quickly from anaesthetic but then the dog presented dyspnea; thus the oxygen supplement was used. The dog

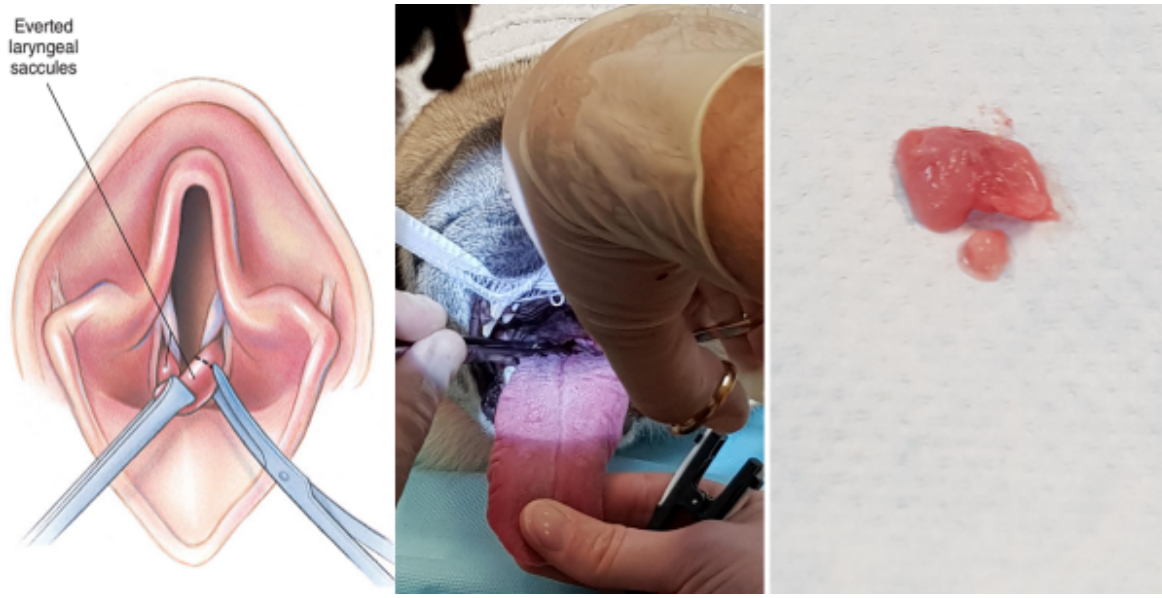

Figure 10. Resection of everted laryngeal saccules (Dog 4). Using Metzenbaum scissor cut off the everted laryngeal saccules (Fossum, 2013).

initially only got inspiratory flow with its mouth held open and tongue out. As recovered from anaesthetic, inspiratory flow was improved but continued to have occasional episodes where the dog retched and moved but then recovered itself. Day 2 in PICU, the dog swallowed the food trial in the morning and then was discharged with omeprazole 10 mg tablet to be given a half twice a day. For the first couple of days, the dog had some difficulty breathing through the nose resulting in self-waking during sleep, causing sleep difficulty. After day 3, the problem was resolved, the dog started to breath well and the gastric reflux seemed to be settled.

3. Discussion

Surgery for BOAS should be planned in the morning, after that, observation is recommended for all day. In post-operative care, surgeries complications include bleeding, swelling, edema, wound problems, aspiration pneumonia and even death. Furthermore, post-operative problems can become more complicated, lead to difficulty breathing and respiratory distress (Fossum, 2013). It is treated, depending on severity, with a combination of sedation, oxygen therapy, intubation, temporary or permanent tracheostomy or mechanical ventilation (Holt et al., 1994; O'Dwyner, 2017). Those sort of major complications occurred in 10% of cases, and a small number of dogs do not survive to discharge, typically due to severe aspiration pneumonia.

4. Conclusions

BOAS is a set of health problems, mainly in the upper respiratory system, that present predominantly in brachycephalic dog breeds. This is the result of congenital malformation of the skull of such breeds leading to various anatomical abnormalities including stenotic nares, tortuous turbinates, caudally displaced maxillae, elongated soft palate, everted laryngeal saccules, and hypoplastic trachea. These abnormalities consequently cause obstruction in nasal cavity, larynx and/or pharynx. Dogs with BOAS may show signs of having respiratory noises, observable nostril stenosis, eating difficulties, regurgitation, sleep dyspnea, sleep apnea, heat intolerance, exercise intolerance and/or collapsing. Correspondingly, the main focus of BOAS surgeries is to unblock the airway. Surgical procedures may include staphylectomy for the case of elongated soft palate, laryngeal sacculectomy for the case of everted laryngeal saccules, alarplasty for the case of stenotic nares, and tonsillectomy for the case of everted/hypertrophy tonsils. Postoperative results from the case studies show the clear improvement in respiratory health in all discussed

cases.

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Application of polypropylene mesh on bilateral perineal hernia: A case study

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ABSTRACT

The protuberance of pelvic viscera is due to the weakness or failure of pelvic diaphragm muscles called a perineal hernia. The aetiology of this disease involved in this process was poorly determined but associated with multi-factors which included gonadal hormone imbalance, rectal abnormalities, prostatic disease, and myopathy. Additionally, the prevalence of middle age and old dogs overwhelmed the young ones due to the change in livelihood and diet. Although there was a certain rate of success in treatment for perineal hernia, this disease had high proportions of recurrence and postoperative complications. This study was conducted at Sasaki Animal Hospital in Ho Chi Minh City to manage this disease by the synthetic mesh implant. A 10-year castrated male Pomeranian (5.2 kg) was referred to clinical examination because of a 4-month complaint of the return of a mass at the perineal area. The owner also reported constipation and hematuria with the presence of pus. Rectal palpation and radiography indicated that the bladder contained in the hernia sac. Ultrasound showed that the bladder contained a large clump of pus. Blood test and antimicrobial-resistant test were also performed by taking the blood and urine with a pus sample, respectively. The ill animals were treated for bacterial infection and then operated to relocate hernia by polypropylene mesh. After 14-day operation, the results were considered satisfactory, which the perineal area returned to normal without any complications. However, the recurrence of this non-infectious disease was reported to range from 10-46%. Therefore, the following health check was necessary to perform twice within a year.

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1. Introduction

Perineal hernia was an acquired disorder caused by the inability of a weak pelvic diaphragm to hold and support pelvic organs. Pelvic disfigurement was clear and occurred either on one or both sides of perineum. Depending on the location of this disfigurement, it was divided into the internal herniation (ventral, inguinal, umbilical and perineal herniation) and the external herniation including diaphragm hernia (Mann et al., 1995; Head & Francis, 2002). Several techniques had been described to correct the perineal hernia. The first method to relocate the herniated organ was traditional herniorrhaphy. However, internal obturator muscle transposition, over the traditional herniorrhaphy in decreasing distortion of the external anal sphincter, was the most common methods due to its success rate (Orsher, 1986). Besides, gluteal muscle transposition, semitendinosus muscle transposition, synthetic implant, and biomaterial were the additional techniques to augment internal obturator muscle transposition. Perineal hernia was

reported more common in dogs than in other animals such as cats, cows. The prevalence of this condition varied from 0.1% to 0.4% (Tobias & Johnston, 2012).

Additionally, the male dog under these circumstances overwhelmed the female one. The ages of the dog were reportedly related to the hernia. The cause of perineal hernia was poorly understood and might be depending on multi-factors. Those factors included congenital predisposition, abnormalities of rectum, imbalance of hormone, prostatic enlargement, which led to strain and the potential feebleness of pelvic diaphragm. Cystitis, urinary tract obstruction, anal sacculitis, perianal inflammation, or diarrhoea could also become a secondary infection (Tobias & Johnston, 2012).

1.1. Atrophy of pelvic diaphragm muscles

The atrophy of pelvic diaphragm, neurogenic origin, was identified in some animals because of the injury of the muscular branches of the pudendal nerve or sacral plexus. It recorded by the electromyogram of the levator ani, coccygeus, and anal sphincter muscles in canine with perineal rupture. The enlargement of prostate gland forcing to the sacral plexus could cause tenesmus (Sjollema et al., 1993).

1.2. Role of rectal abnormalities

Rectal abnormalities reported existing together with the perineal hernia. There were three common recognizations of this condition, which were derivation, dilation and diverticulum (Krahwinkel, 1983). It was also theoretical that these conditions were the results of perineal herniation rather than the causes. Nonetheless, excessive straining resulted from obstruction and diverticulum of the rectum.

1.3. Role of hormones

Androgen in the intact male dog thought to become an essential role in perineal hernia. The testosterone function was poorly unknown its reaction. Some authors suggested cause the dilation of the pelvic diaphragm, whereas the concentration of the testosterone and estradiol-17 were the same in those who had or no perineal hernia (Mann et al., 1989). According to another study, the sensitivity and quantity of the androgen receptors of the pelvic diaphragm muscles were less in castrated and intact dogs with perineal hernias, which compared with the norma group (Mann et al., 1995).

Relaxin was the hormone that synthesized primarily by the prostate gland in male dog whereas in female ones was by corpus luteum, the breast and the placenta during the pregnancy (Gert et al., 2005). Despite the poor understanding of the role of relaxin, it identified that the relaxin receptors within the pelvic diaphragm muscle expressed higher in the dog with perineal hernia compared with its normal (Merchav et al., 2005). The development of the perineal rupture might be associated with the relaxin because of the presence of the cystic hypertrophy of canine prostate gland, which both located adjacently and contained a high concentration of relaxin (Niebauer et al., 2005).

Rectal prolapse, bladder retroflexion and prostatic displacement were complicated in the surgical treatment of perineal hernia, which required the advanced procedures, such as colopexy, cystopexy, and vasopexy (Maute et al., 2003; Bongartz et al., 2005). This clinical case report would discuss the surgical techniques by using polypropylene mesh, recurrence, and the complication after the operation.

2. Materials and Methods

2.1. History

A 10-year castrated and vaccinated mixed pomeranian called Carrot was brought to Sasaki hospital on September 5th, 2019. The owner reported that the problem was urinary bladder herniation and experienced the surgery in another clinic 4 months ago. However, only three months after, the clinical signs were appeared again but more severe, which were hematuria with pus and bilateral perineal swelling. Faecal incontinence was also presented.

2.2. Clinical examination

His body condition was not reasonable. First, his temperature was 38.9°C, a little bit high than the normal range, and his body weight was 5.2 kg. The mucous membranes were healthy. The cardiac and lung sound was quite clear without any abnormalities. The abdominal palpation fell to find anything except the urinary bladder. The integument area around the anus was red-

Table 1. Complete blood count results

Criteria	Range	Results
RBC[1]	550-850 x 10^4 cells/µL	889 x 104 cells/µL
Ht[2]	37-55 %	56.4 %
Hb[3]	12-18 g/µL	20.7 g/µL
WBC[4]	6000-17000 cells/µL	25100 cells/µL
MCV[5]	65-72 fL	63.4 fL
MCHC[6]	32-37 g/100 mL	36.7 g/100 mL
MCH[7]	19.5-34.4 g/100 mL	23.3 g/100 mL
PLT[8]	17.9-51 x10^4/µL	29.1 x104/µL

[1]Red blood cells, [2]hematocrit, [3]hemoglobin, [4]white blood cells, [5]mean cell volume, [6]mean corpuscular hemoglobin concentration, [7]mean corpuscular hemoglobin, [8]platelets.

Table 2. Serum biochemistry test results

Criteria	Range	Results
BUN[1]	9.2-29.2 mg/dL	68.1 mg/dL
CRE[2]	0.4-1.4 mg/dL	1.09 mg/dL
T-CHO[3]	111-312 mg/dL	261 mg/dL
GLU[4]	75-128 mg/dL	138 mg/dL
GPT[5]	17-78 mg/dL	62 mg/dL
ALP[6]	47-254 mg/dL	271 mg/dL

[1]Blood urea nitrogen, [2]creatinine, [3]total-cholesterol, [4]glucose, [5]glutamic pyruvic transaminase, [6]alkaline phosphatase

ness, swelling. His locomotion also had a problem. Then Dr. Kamijo did the digital rectal examination, which used the index finger passing efficiently into his dilated segment of the rectum when angled lateral and caudal to the anus. After finishing the physical examination, blood and urine with pus took for biochemical and antibiotic resistance test, respectively.

2.3. Diagnostic image

According to the radiography, his urinary bladder herniated into the hernial sacculation. Fortunately, there was no urinary bladder retroflexion; however, plenty of faeces defecated in the rectum. In his urinary bladder, urolithiasis did not present, which could not summarize there were no urinary bladder crystals because some of the crystals did not appear under X-rays. Subsequently, the abdominal ultrasound indicated that a clump of pus existed in the urinary bladder without any crystals (Figure 1).

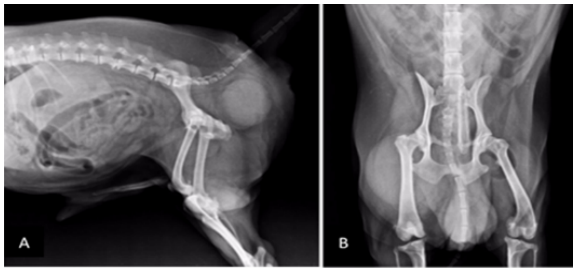

Figure 1. Lateral ventral view (A) and dorsoventral view (B) of Carrot with a urinary bladder in the perineal sac.

2.4. Laboratory test

According to the results, his WBC was approximately 1.5 times higher than average (Table 1). In other words, the acute inflammatory process happened caused the increase in WBCs margination and migration (Meyer et al., 1992). Cytokines related to an acute inflammatory procedure enhanced the bone marrow proliferation to release the WBCs, which was eliminating the invading microbes (Cunningham & Klein, 2007). In the biochemistry, BUN accurately increased 3 times than normal range (Table 2). This change was not the kidney disease because of the routine ultrasound of Carrot's kidney. The reason for this were gastrointestine.

The result of the clump of pus sent to Nam Khoa Biotek company for antimicrobial the resistant test was Klebsiella pneumonia, which was named by ISD 14 GNR. This system included 14

small reactions that could identify the name of bacteria. This bacteria was gram-negative rod-shaped bacterium as well as the opportunistic invader of the urinary tract in dogs and cats (Quinn et al., 2011). Then the antimicrobial resistance test was performed.

According to the results, there were only three sensitive antibiotics which were Amikacin, Imipenem and Cefoxitin (Table 3). This bacterium secreted extended-spectrum beta-lactamase enzyme, which meant the resistance to all the group of Cephalosporin even the 4th generation of this group (Tran, 2016). Depending on the AMR result, the veterinarian could choose the right antibiotics for Carrot.

Table 3. Antimicrobial resistance test results

ESBL[1]	+
Amoxicillin/ Clavulanic acid	Resistant
Sulfamethoxazole/ Trimethoprim	Resistant
Gentamicin	Resistant
Cefuroxime	Resistant
Amikacin	Sensitive
Cefepime	Resistant
Imipenem	Sensitive
Cefoxitin	Sensitive
Ampicillin	Resistant
Cefotaxime	Resistant
Piperacillin/tazobactam	Resistant
Ciprofloxacin	Resistant
Ceftazidime	Resistant
Tetracycline	Resistant

[1]Extended spectrum beta lactamase

2.5. Diagnosis

Direct rectal examination of the animal could identify several causes of the diaphragm herniation, which involved tissue disease in the perineal area, inflammation of the perineal area, inflammation of the urinary tract and complete health profile of the client to differentiate to the abscess, tumour, hematoma or cyst. Radiography, ultrasounds and blood test were used to view the damaged area. Additional tests would be necessary to indicate the underlying cause and identifying treatment. All of these things which had done made the diagnosis correctly to perineal herniation. Normally, the prognosis of perineal hernia was fully acceptable for most of the cases; however, there was 10-15% of recurrence of another perineal hernia with in a year (Dwyer, 2018).

2.6. Bacterial infectious treatment

Urinary bladder catheterised to eliminate the aggression of pus and blood as much as possible. Besides Amikacin was chosen to treat this bacterial infection with 15 mg/kg, IV, SID and prednisolone with 1 mg/kg, PO, SID for the first three days (Ian, 2017). Additionally, lactulose also used as a laxative in small animal with 0.5-1 mL/kg, PO, BID (Plumb, 2008). However, the aminoglycoside group had some side effects, including nephrotoxicity and ototoxicity as well as its efficiency fell to work in this bacterial infection. Therefore, another antibiotic, imipenem/cilastatin, became a choice in this case. Imipenem was the member of the carbapenem group with a broad-spectrum bactericidal agent with 10 mg/kg, IV, TID (Plumb, 2008). At the end of the therapy, he acted like reasonable condition. Subsequently, the surgery was hold to relocate the herniated organs (Table 4 & 5).

2.7. Anaesthetic procedure

Carrot was asked to give no food and drink after 9 pm until the day after before the surgery. Then he was given the intravenous catheter and injected atropine sulfate, an anticholinergic agent with 0.04 mg/kg, SC, was used for the pre-anaesthetic stage which meant 15 min before the surgery. The medicine for induction was propofol with 4 mg/kg, while the antimicrobial and anti-inflammatory agent used was cefotaxime with 27 mg/kg, IV, TID and meloxicam 0.2 mg/kg, SC, SID in the morning, respectively (Ian, 2017).

After that, vets would do the tracheal intubation by the tools, first for the maintenance stage by sevoflurane. This halothane did not stimulate the respiratory system with proper muscular dilation, fast and gentle anaesthesia as well as quicker recovery than isoflurane used before in the hospital. However, there were some side effects which would be unstable when contacting with CO_2 in the anaesthetic machine, which was decomposed into vinyl ether called "Compound A". This compound was reported for nephrotoxicity and neurotoxicity (Tran, 2016). The monitoring would be noticed by the anaesthetic groups. Then the nurses shaved fur around the site of the surgery area and disinfected twice. The first time was with chlorhexidine shampoo and the second time with povidone scrub, then leaving it dry naturally, covering it by sterile drapes and clipping

Table 4. Complete blood count results after 20-day treatment

Criteria	Range	Results
RBC	550-850 x 10^4 cells/µL	727 x 10^4 cells/µL
Ht	37-55%	45.6%
Hb	12-18 g/µL	16.6 g/µL
WBC	6000-17000 cells/µL	12300 cells/µL
MCV	65-72 fL	62.7 fL
MCHC	32-37 g/100 mL	36.4 g/100 mL
MCH	19.5-34.4 g/100 mL	22.8 g/100 mL
PLT	17.9-51 x10^4/µL	33.2 x10^4/µL

Table 5. Serum biochemistry test results after 20-day treatment

Criteria	Range	Results
BUN	9.2-29.2 mg/dL	20.9 mg/dL
CRE	0.4-1.4 mg/dL	0.5 mg/dL
T-CHO	111-312 mg/dL	302 mg/dL
LIP	0-160 U/L	36 mg/dL
GPT	17-78 mg/dL	82 mg/dL
ALP	47-254 mg/dL	249 mg/dL

the anus by the skin staplers.

2.8. Surgical procedure

From over the hernia through the skin began near the tail base made an incision, which extended ventrally to midway between ischial tuberosity and pubis. To exteriorize the hernia sac, this incision was made as a slight curve so that its midpoint was directed away from the anus then dissected the subcutaneous tissues.The incision was made through the hernial sac which followed the same line as the skin incision then press gently and firmly to relocate herniated organs into the pelvic or abdominal cavity (Figure 2).

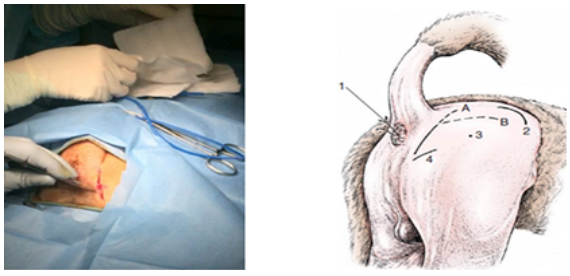

Figure 2. Preparation of the surgical area. Incision traditional perineal method (A); superficial gluteal transposition (B). 1. staple the anus; 2. iliac crest; 3. femoral greater trochanter; 4. ischial tuberosity (Bellenger and Canfield 2003).

The perineal structures were exposed due to the reduction of hernia. The coccygeus and levator ani muscles border dorsolateral defect. The rectum and external anal sphincter bordered medial defect. The lateral site was the sacrotuberous ligament, a thick, fibrous cord extending from the ischiatic tuberosity to the sacrum. The internal obturator muscle was bordered the hernia ventrally, lying and firmly bounding to the ischium between the ischial arch and obturator foramen. Repeat to the opposite side then using the aneurysm needle to check obturator foramen of both sites (Figure 3).

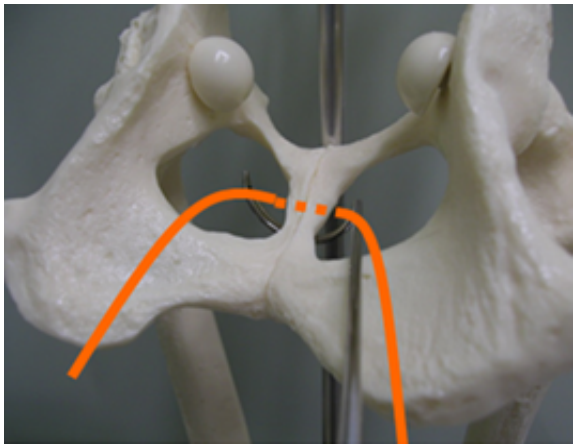

Figure 3. The aneurysm would follow the orange line.

A piece of polypropylene mesh was tightened to the catheter by the polypropylene suture, which would be immobilized with the aneurysm needle. This mesh fitted into the pararectal fossa. The mesh was inserted with the catheter-directed cranially and advanced until the edges opposite the fold are adjacent to the anal sphincter. Then remove the aneurysm needle (Figure 4).

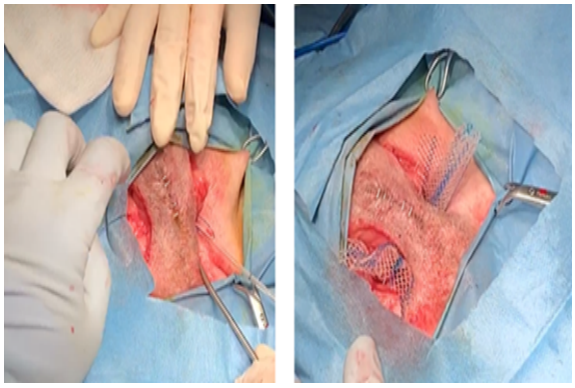

Figure 4. Pulling the suture with the catheter to the other side before removing the catheter.

By using 2-0 monofilament, polypropylene sutures placed dorsally and laterally in the coccygeus muscle, laterally in the sacrotuberous ligament, ventrally in the fascia of the internal obturator, and medially into the levator ani muscle and the external anal sphincter with the polypropylene mesh. After the sutures are in place, the surgeon tied to adipose tissues without excessive tension, and the surgical wound was closed by simple interrupted suture (Figure 5).

After the sutures were in place, the surgeon tied to appose tissues without excessive tension, and the surgical wound was closed by simple interrupted suture. Immediately, the staple-suture was removed and rectal palpation to check any abnormalities (Figure 6).

2.9. Post-operative care

Immediately, the staple-suture was removed and rectal palpation to check any abnormalities. Carrot was advised to stay at the hospital for seven days before coming back to home. An Elizabethan collar was worn to avoid licking and chewing at the incision site until the suture remove. The inflammatory agent and broad-spectrum antibiotic were continuously used during the post-operative period. The wet food was also performed to soften the stool.

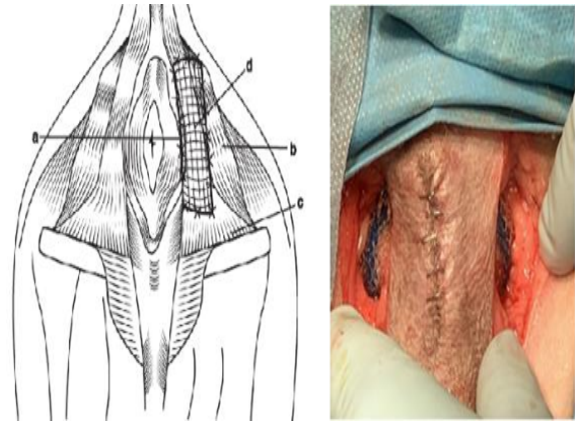

Figure 5. Prosthetic implantation. A polypropylene suture (d) was placed dorsally and laterally in the coccygeus muscle (b), ventrally to the fascia of the internal obturator muscle (c), and medially into the external anal sphincter (a) and levator ani muscle with the polypropylene mesh (Gill et al., 2018).

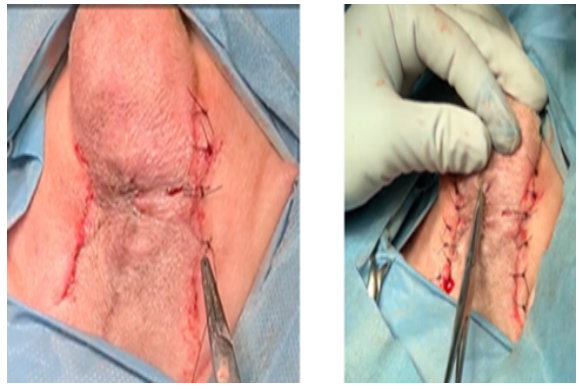

Figure 6. Close the surgical area by the simple interrupted pattern and take the stapler out.

2.10. Results

After two weeks, Carrot came back to have a health examination. The wound was healed, and the suture was taken out. Subsequently, the owner reported Carrot condition was good, and there was no appearance of the recurrence after two months and until now. Compare to the prognosis above, there was a successfully healing in this case without any complications or reccurrence (Figure 7).

3. Discussion

Polypropylene mesh was either used alone or adjusted with other procedure for the correction of perineal hernia (Clarke, 1989; Vnuk et

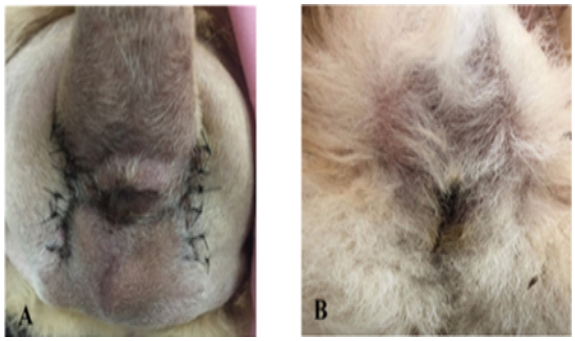

Figure 7. After 14-day operation.

al., 2006). One study also indicated that the success rate of these techniques led to approximately 92% (Clarke, 1989). It was reported that the advantages of this mesh were its strength and easy management. Although this mesh was applied in the closure of thoracic, abdominal wall, the skull, perineal, diaphragmatic and tracheal collapse in dogs, few studies evaluated the complications which were related to the implantation of polypropylene mesh (Bowman et al., 1998). The design of polypropylene knit with uneven pore sizes ranging from 200 µm to 800 µm was required for ingrowth of vascularized connective tissue and immediately infiltrated by capillaries and fibroblasts (Chvapil et al., 1972).

Wound breakdown and infection were the most common postoperative complications in dogs after correction of perineal deformation not presenting the polypropylene mesh, with the range of 6.4% to 45% (Burrow & Harvey, 1973; Orsher, 1986). The application of polypropylene mesh was able to increase the risk of infection (Brown et al., 1985). Polypropylene mesh had the structure of nonabsorbable with polypropylene monofilament. This structure was believed to prevent bacteria from being ambushed within the fibres, which made this mesh different from others to become infection (Fox et al., 1988; Trostle & Rosin, 1994). According to Kelly & Behrman (2002), in human, the clean-contaminated and contaminated operation could be performed with prosthetic mesh in the would-related morbidity and mortality. Nevertheless, fistula formation and mesh expulsion happened after months to years later (Falagas & Kasiakou, 2005).

During the treatment, prophylactics antibiotics were used and cleaning of the perineal region with chlorhexidine 2% owing to the high incidence of contamination of faeces and from the shelter. It was indicated that there was an increase in the rate of postoperative infection in dogs that passed the clean surgical procedures and received prophylactic antibiotics compared with others receiving without antibiotics (Usher & Gannon, 1959). Cleaning around the surgical wound could lead to inhibit the wound healing.

Recurrence was also observed as the most popular complication of perineal hernia correction. Two critical factors that played a prominent role in recurrence, which was the status of perineal tissues and the degree of atrophy of muscular pelvic diaphragm (Lee et al., 2012). Hernia recurrence in this study was depended on the observation of swelling perineal integument and the presence of clinical such as tenesmus. It was difficult that recurrence was accurately determined if no related to clinical signs were noted after the swelling of the perineum. However, there was no appearance of recurrence in this case 8 months ago.

4. Conclusions

Perineal herniations were commonly referred to as the weakness of pelvic diaphragm muscles, which were an old dog. Carrot, a 10-year dog, showed us how perineal hernia relocated and what procedure had to apply to bring more welfare for a companion animal in this modern life. At Sasaki Animal Hospital, new technique and equipment first applied in this case. These could increase in animal health and welfare. Because of the probability of this condition happening on the male dog more than female, castration was the choice to reduce the recurrence. Postoperative care also played an essential role in the therapy, although there were some incident complications, which were infection from the surrounding area, nerve damage, other anal or rectal problems. This was an effective and less invasive procedure. Although the material might be costly, those techniques were uncomplicated, high success rate and low recurrence.

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Seroprevalence against classical swine fever virus in vaccinated pigs in Ho Chi Minh City

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ABSTRACT

The aim of this study was to survey the serological response to classical swine fever disease in vaccinated pigs in Cu Chi, Ho Chi Minh City. By using the PrioCHECK® CSFV Ab 2.0 ELISA test kit to detect antibodies against CSF in 410 vaccinated pigs and IDEXX CSFV Ag Serum Plus Test to detect the E^{rns} protein of the CSFV in pigs without antibodies against CSFV. Results showed that the overall seroprevalence observed in vaccinated pigs in other Farms varied from 70% - 100% ($P < 0.05$), but in Farm 5, no pigs produced a positive humoral response against CSFV were found. The highest seroprevalence of antibodies against CSFV was found in Farms with a herd size of $\geq$ 1000 - $<$ 6000 animals (91.26%) and the lowest was a Farms with less than 1000 animals (51.81%). The highest ratio of positive pigs for antibodies against CSFV belonging to Group of $>$ 40 - $\leq$ 60 days post-vaccination was 98.36%; and the lowest rate was found in Group of 30 - $\leq$ 40 days post-vaccination (51.96%). That grower pigs had the highest proportion of positive pigs for antibodies against CSFV accounting for 81.40%; next, the proportion of positive sows was 73.24%. Significant differences in the seroprevalence observed in vaccinated pigs across herd size, days post-vaccination, type of pigs ($P < 0.05$). In this study, no pig was found to contain CSFV antigen.

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1. Introduction

Classical swine fever (CSF) also known as hog cholera, is one of the most important viral diseases of domestic pigs and wild boar. In infected pigs, the CSF virus causes one of several forms (acute, chronic, or prenatal) and can result in high morbidity and mortality in swine. In 1997, an outbreak of CSF occurred in the Netherlands and caused more than €2.5 billion (EURO) in losses and more than 11 million pigs were destroyed (Meuwissen et al., 1999). CSF disease still exists and causes economic losses to the swine industry in many countries in Asia, Latin America and Europe (WAHID, 2015). Strategies to control classical swine fever virus (CSFV) mainly consist of stamping out policy (non-vaccination) and a systematic prophylactic vaccination (Huang et al., 2014). In Vietnam, since 1980, with systemic prophylactic vaccination, severe CSF outbreaks have been controlled in pig farms. Therefore, the development of management programs, proactive vaccination, vaccination control, post-vaccination surveillance for CSF disease is essential to reduce the damage caused by the disease. Especially in Ho Chi Minh City, the CSF vaccine has been recommended in the routine vaccination program for animals according to Decision no. 07/2016/TT-BNNPTNT about the national program of controlling CSF disease issued in 2016

(MARD, 2016). For that reason, the purpose of this study is to assess the serological response to classical swine fever disease in vaccinated pigs in Cu Chi, Ho Chi Minh City.

2. Materials and Methods

Total 410 pigs from 7 pig farms in 6 wards (An Nhon Tay, Hoa Phu, Nhuan Duc, Pham Van Coi, Phu My Hung, Trung Lap Thuong) of Cu Chi district, Ho Chi Minh city were selected at random and approved for use in this study by Animal Health Laboratory and Treatment Division, Subdepartment of Animal Health and Husbandry of Ho Chi Minh City. A total of 410 serum samples were originally collected as part of on-going annual disease investigations. In each farm, depending on herd size and the permission of owners, 5 – 100 pigs were collected randomly for blood sampling. All pigs were vaccinated Coglapest (Ceva, Hungary) or Dich Ta Heo (Navetco, Vietnam) against CSFV and blood samples were collected from 30 to more than 60 days post-vaccination. All serum samples were tested for the presence of antibody against CSFV; then, a total of 108 serum samples were negative for CSF antibodies would be detected E^{rns} antigen by using IDEXX CSFV Ag Serum Plus Test kit. PrioCHECK$^{\text{ő}}$ CSFV Ab 2.0 (Prionics Lelystad B.V. Netherlands) is used to detect antibodies against CSFV and IDEXX CSFV Ag Serum Plus Test is used for the presence of E^{rns} protein of CSFV. The ELISA procedures of this study were performed according to the manufacturer's recommendations. Descriptive analysis was performed and reported as a mean value. Chi-square test was used to compare the difference in proportions of seroprevalence of age groups, breeds, and other variables. The difference level $P < 0.05$ will be considered as significant difference.

3. Results and Discussion

The overall seroprevalence observed in vaccinated pigs was 73.66% (302/410). Compared with previous studies, the higher seroprevalences of vaccinated pigs in the study by Lam Hoang Kiet but lower seroprevalences of vaccinated pigs in the study by Nguyen Le Thanh were 65,63% and 75.66%, respectively (Lam, 2009; Nguyen, 2018). In this study, no pig was positive for the presence of antibodies against CSFV in Farm 5 was found. There were significant differences in the seroprevalence observed in vaccinated pigs across Farms ($P < 0.05$). In this study, except Farm 5, six other Farms met the demand of the Sub-department of Animal Health Ho Chi Minh scheme (MARD, 2016), which required the ratio of a positive result against CSFV antibody of a herd must be greater than 70%. According to Blome et al. (2017), CSF vaccination was still in use to reduce the disease burden in endemically affected countries. As mentioned above, all pigs vaccinated Coglapest (Ceva, Hungary) or Dich Ta Heo (Navetco, Vietnam) against CSFV were selected randomly for the detection the seroprevalence of pigs from CSFV; therefore, it is hard to explain why Farm 5 had no positive pigs for antibodies against CSFV. A previous study suggested that antibody responses against the CSF vaccine were significantly reduced in *Trypanosoma evansi* infected pigs as compared to uninfected pigs. This immunosuppression might explain the accounts of poor protection of CSF vaccinated pigs reported in *T. evansi endemic* areas of Vietnam (Holland et al., 2003). It is likely that poor handling and malpractice in CSF vaccination in Farm 5 occurred and this leads to the failure in CSF vaccination on this Farm. In reality, the owners were responsible for vaccination programs in a pig farm and the information about these pigs such as vaccination programs was collected by using questionnaire lists. Furthermore, maternally derived antibody was the most common cause of CSFV vaccination failure, particularly in highly endemic areas (Suradhat et al., 2007), and therefore piglets that have circulating maternal antibody may not seroconvert when vaccinated.

A significant difference in positive pigs among the three groups of herd sizes was found ($P < 0.05$) in Table 1. According to Moening (2000) and Guo et al. (2011), the percentage of positive pigs for antibodies against CSFV influenced by many factors, such as the type of antigen in the vaccine, the integrity of the vaccine, age of pigs when vaccinated (associated with maternal antibody), application of vaccination by officers, environmental conditions and pig health condition. The success of vaccination programs is determined by the formation of protective antibodies in pigs (Ratundima et al., 2012). It is likely that Farms with herd sizes over 1000 animals applied proper hygienic and disease prevention procedures.

Based on days post-vaccination, there was a significant difference about the seroprevalence

Table 1. The seroprevalence of antibodies against classical swine fever virus

		Number of samples	Number of positive samples	Ratio (%)
Farm	1	61	60	98.36
	2	61	61	100.00
	3	61	46	75.41
	4	100	70	70.00
	5	61	0	0.00
	6	5	5	100.00
	7	61	60	98.36
Herd sizes (animal)	< 1000	127	65	51.18
	≥ 1000 - < 6000	183	167	91.26
	≥ 6000	100	70	70.00
Time of vaccination (days)	30 - ≤ 40	127	66	51.96
	> 40 - ≤ 60	122	120	98.36
	> 60 - ≤ 90	161	116	72.04
Type of pigs	Sow	71	52	73.24
	Gilt	64	43	67.19
	Boar	60	32	53.33
	Grower	215	175	81.40

observed in pigs after vaccination ($P < 0.05$) in this study. In North Central provinces, Bui (2001) reported that the proportions of positive pigs for antibodies against CSFV after 21 days, 3 months and 6 months post-vaccination were 84.66%, 78.88%, and 35.15%, respectively. Precausta et al. (1983); Terpstra et al. (1990) and Dahle and Liess (1995) reported that the vaccination with a C-strain CSF vaccine-induced neutralizing antibodies that usually appear about 2 weeks after vaccination and increase until at least 4–12 weeks; they can persist for many years after (a single) vaccination. However, Terpstra and Tielen (1976) also indicated that some pigs did not produce antibodies against CSFV after vaccination. Most of the sows that had been vaccinated once 1–3 years earlier did not respond with an increase in antibody titre upon a second vaccination (Terpstra & Tielen, 1976). When pigs are vaccinated in the presence of maternal antibodies, the formation of neutralizing antibodies is markedly inhibited. However, when such pigs were vaccinated a second time, many animals did show a rise in antibody titre after the second vaccination (Terpstra & Wensvoort, 1987). Likewise, Bui (2001) also confirmed that the appearance of virus-neutralizing serum antibodies could be inhibited by the presence of maternal antibodies in pigs being vaccinated against CSFV.

Furthermore, significant differences in seroprevalence observed in vaccinated pigs were found among types of the pig ($P < 0.05$). In previous studies, the ratio of positive sows for antibodies against CSFV (70.97%) was higher than that of growers (53.62%) (Lam Hoang Kiet, 2009). Likewise, the seroprevalence of antibodies against CSFV in sows and growers was 89.05% and 52.21%, respectively (Nguyen, 2013). Huynh (2018) reported that the proportion of positive sows for antibody against CSFV (91.33%) was higher than that of growers (78.80%).

Of 108 negative pigs for antibodies against CSFV tested to screen for CSFV-specific antigen by using IDEXX CSFV Ag Serum Plus Test, no pig was found to contain CSFV antigen. This result was consistent with the result of the Ho Chi Minh Department of Animal Health in Cu Chi district in 2018, of 180 serum samples were analyzed for the presence of CSF antigen, no pig was found to contain CSFV antigen (Department of Animal Health, 2018). However, future vaccine developments should be stronger focused on a tailored DIVA (differentiating infected from vaccinated animals) assay to identify the differentiation between infected and vaccinated animals.

4. Conclusion

In conclusion, of seven farms selected, the highest percentages of positive pigs for antibodies

against CSFV in Farm 1 and Farm 2 of An Nhon Tay, Farm 6 of Hoa Phu and farm 7 of Trung Lap Thuong communes were over 95% and no positive pigs in Farm 5 of Phu My Hung commune. Significant differences about the seroprevalence were observed in vaccinated pigs across herd size, days post-vaccination, type of pigs. No pig was found to contain CSFV antigen.

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Serological survey on Leptospirosis of cattle in Cu Chi, Ho Chi Minh City

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ABSTRACT

Leptospirosis is a zoonotic disease of global importance, especially in tropical countries. The purpose of this study to evaluate the seroprevalence of *Leptospira* in cattle in Ho Chi Minh city. Serum samples from 446 animals were tested by microscopic agglutination test (MAT) using a panel of 24 *Leptospira* serovars kits provided by Ho Chi Minh City Pasteur Institute. Results showed that seroprevalence of *Leptospira* at herd-level was 61.54%; and at individual-level was 31.17%. In which, the percentage of beef cattle (29.77%) infected with *Leptospira* was lower than that of dairy cattle (31.75%). Cattle from 2 to 3 years of age had the highest rate of *Leptospira* infection in was 37. 72%. According to herd size, the results found that medium-scale farming (from 25 to 50 animals) had the lowest seroprevalence rate with *Leptospira* spp. (28.65%). No significant differences were found among aged groups of cattle. There were a totally 7 serovars that were detected in cattle, in which, the two most popular serovars were L. Hardjo bovis (31.37%) and L. Hebdomadis (30.26%). There were twelve animals infected with four *Leptospira* serovars (8.63%); meanwhile, the majority of animals infected with one serovar were 42.45%. The ratio of positive animals at antibody titer of 1:200 was 53.70%. These results indicated a very high exposure of Cu Chi cattle to *Leptospira* spp. which consequently posed a definite risk for people working with cattle acquiring this zoonotic infection.

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1. Introduction

Leptospirosis is a neglected zoonotic disease and can be devastating to both human and animal health globally. It is caused by pathogenic bacteria of the *Leptospira* genus from the family Leptospiraceae (Levett, 2001). This disease is mostly endemic in humid tropical or subtropical countries. Animals or humans can be infected with this pathogen from exposure to contaminated reservoirs such as carrier mammals, contamination of soil and water. Domestic animals act as a reservoir for pathogenic *Leptospira* represents a significant health risk to a wide range of workers, veterinarians and slaughterhouse workers (Adler & Adela, 2010). It was estimated that seven to ten million people were infected by leptospirosis per year and almost 58,900 deaths occurred per year (Costa et al., 2015). *Leptospira* was first identified in 1930 and is known to be endemic for leptospirosis with a peak during the rainy season (Laras et al., 2002). In dairy cattle, though Leptospirosis is sub-clinical or mild in most cases, severe illness can sometimes end fatally, a series of clinical signs such as abortion, mastitis, loss of milk are found. According to WHO (2011), "there is little research on the economic impact of leptospirosis, and information is lacking about the societal costs of the disease, including the costs of health care, lost productivity

Table 1. The seroprevalence of *Leptospira* at herd-level by region

Regions	Number of households	Number of households contained positive cattle	Ratio (%)
An Nhon Tay	7	2	28.57
An Phu	10	7	70.00
Binh My	3	2	66.67
Hoa Phu	5	0	0.00
Nhuan Duc	3	3	100.00
Pham Van Coi	1	1	100.00
Phu Hoa Đong	6	2	33.33
Phu My Hung	5	2	40.00
Phuoc Hiep	2	1	50.00
Phuoc Thanh	5	5	100.00
Phuoc Vinh An	4	0	0.00
Tan Thanh Dong	23	17	73.91
Tan Thanh Tay	4	2	50.00
Thai My	2	2	100.00
Trung An	5	5	100.00
Trung Lap Ha	2	2	100.00
Trung Lap Thuong	4	3	75.00
Total	91	56	61.54

caused by sequelae, and death of livestock". However, it showed a significant public health problem and the economic losses result from infertility, abortion, poor milk yield (Lloyd-Smith et al., 2007). The aim of this study to assess the seroprevalence of *Leptospira* in cattle in Ho Chi Minh City. This information will be reference documents to determine the strategy of leptospirosis prevention and control in humans and animals in Ho Chi Minh City.

2. Materials and Methods

Serum samples from 446 cattle were collected from 90 households in Cu Chi district, Ho Chi Minh city from July to October, 2020. Information about the cattle including breed, age, gender, day of vaccination and day of sample collection were recorded. There are about 30 - 40% cattle of total number of cattle per household chosen randomly to collect blood sample. Then, blood samples were left to clot naturally at room temperature. Sera from those tubes after blood clotting were centrifuged to be completely clear and free of hemolyzed blood cells. All sera were labeled with the animal identification number before being stored at -70°C until testing using MAT. Total of twenty four serovars were chosen based on the test kit provided by the Pasteur Institute, Ho Chi Minh City. Each tube of *Leptospira* contained approximately 2 x 10^8 leptospires/mL. The principle of the MAT reaction is the agglutination reaction occurred between the surface of live *Leptospira* and the specific antibody in the test serum against *Leptospira*. The degree of agglutination of the antigen-antibody complex was assessed under a dark-field microscope. This test detects antibodies specific for the *Leptospira* bacteria and is the World Health Organization gold standard test to diagnose leptospirosis.

For data analysis, the overall seroprevalence, prevalence of each serovar, and 95% confidence intervals were calculated by utilizing Excel 2016. Chi-square tests were used to compare the differences in the proportion of seroprevalence of each serovar according to herd size, age group and other variables according to the Minitab 16.0 software.

3. Results and Discussion

According to Table 1 the result showed that the highest percentage of households with cattle infected with *Leptospira* spp. was 100% in Nhuan Duc, Pham Van Coi, Phuoc Thanh, Thai My, Trung An and Trung Lap Ha. The statistical difference was found in the proportion of households with infected animals among regions ($P < 0.05$). Previously using an agglutination test with the

Table 2. The seroprevalence of *Leptospira* spp. in cattle

		Number of tested samples	Seropositive samples (a titer ≥ 1/100 for any serovars)	Ratio (%)
Regions	An Nhon Tay	32	4	12.50
	An Phu	70	25	35.71
	Binh My	11	3	27.27
	Hoa Phu	23	0	0.00
	Nhuan Duc	14	7	50.00
	Pham Van Coi	3	2	66.67
	Phu Hoa Đong	28	3	10.71
	Phu My Hung	18	4	22.22
	Phuoc Hiep	10	2	20.00
	Phuoc Thanh	26	12	46.15
	Phuoc Vinh An	14	0	0.00
	Tan Thanh Dong	117	48	41.03
	Tan Thanh Tay	19	6	31.58
	Thai My	8	5	62.50
	Trung An	25	5	20.00
	Trung Lap Ha	9	4	44.44
	Trung Lap Thuong	19	9	47.37
Herd size (heads)	< 25	218	68	31.19
	≥ 25 - < 50	171	49	28.65
	≥ 50	57	22	38.60
Types of cattle	Beef cattle	131	39	29.77
	Dairy cattle	315	100	31.75
Age (years)	< 1	34	11	32.35
	≥ 1 - ≤ 2	137	33	24.09
	> 2 - ≤ 3	114	43	37.72
	> 3 - ≤ 4	85	29	34.18
	> 4	76	23	30.26

same panel of *Leptospira* spp. strains provided by the Pasteur Institute, Ho Chi Minh City, this result was consistent with the results of Pham (2016), the rate of household with cattle infected with *Leptospira* was 61.22% in 6 districts (Cu Chi, Binh Chanh, Hoc Mon, Thu Duc, 9 and 12) of Ho Chi Minh City.

In Table 2, of 446 serum samples were tested by MAT method, there were 139 seropositive samples with a titer $\geq 1/100$ for any serovars were found, accounting for 31.17%. No significant differences were found among groups with herd size and type of cattle ($P > 0.05$). Risk factors, including the use of well or stream water, minding livestock, walking barefoot, and the presence of rats and cats at home, were associated with being exposed to *Leptospira* (Ganoza et al., 2006). It is likely related to the increased risk of exposure, transmission and persistence of infections in larger intensive herds. Lilenbaum & Santos (1996) reported that there was a positive association between herd size and the presence of cattle infected with serovar Hardjo. However, there was limited understanding regarding seroprevalence and transmission of pathogenic *Leptospira* in herds comprised of both dairy and beef cattle (Martins et al., 2011).

Moreover, in accordance with other studies, *L.* Hebdomadis (30.26%) and *L.* Hardjo hardjo bovis (31.37%) were also the most popular leptospires in cattle in Ho Chi Minh City (Table 3) and the ratios were 23.10% and 18.05%, respectively (Tran, 2016). In Northern Ireland, Ellis and others made the remarkable observation that serovar hardjo was present in 41.6% of 245 randomly selected aborted bovine fetuses (Ellis et

Table 3. Serovar distribution among 131 seropositive cattle determined by positive MAT (titer ≥ 1:100)

Serovars tested	Number of cases	Frequency (%)
L. Autumnalis	6	2.21
L. Bataviae	3	1.10
L. Hardjo Hardjo-bovis	85	31.37
L. Hardjo Hardjoprajitno	51	18.82
L. Hebdomadis	82	30.26
L. Tarassovi	35	12.92
L. Vughia	9	3.32

Table 4. The percentage of positive samples reacting with one or more serovars

Number of positive serovars	Seropositive samples (a titer ≥ 1/100 for any serovars)	Ratio (%)
1	59	42.45
2	40	28.78
3	28	20.14
4	12	8.63
Total	139	100.00

Table 5. Antibody titers rates are agglutinated to *Leptospira*

Antibody titer mean value	1/100	1/200	1/400	1/800	Total
Number of MAT positive	42	145	72	11	270
%	15.56	53.70	26.67	4.00	100.00

al., 1982). According to Radostits et al., (2000) reported that serovar Hardjo is usually the most-prevalent in the cattle world widely and is considered the most adapted to cattle. This result was in agreement with the study of Bahlibi (2015), the prevalence was significantly highest in cattle with greater than 6 year's age group (29.7%), followed by 3 to 6 years age group (23.2%); while the lowest rate was found in cattle less than three years old (6.7%). It was likely that the older animals were not at greater risk of infection by this organism, but this may be a reflection of the long duration and persistence of antibodies in the animals and a longer period of exposure.

Table 4 showed the majority (42.45%) of the cattle which was tested positive for MAT results showed a reaction to only one serovar, and the rest reacted multiple serovars. The highest numbers of serovars detected were four serovars in 12 animals (8.63%) (Table 4). According to Cousins et al. (1989), each serovar is adapted to a particular maintenance host, although they may cause disease in any mammalian species. Cattle are maintenance hosts for *L. borgpetersenii* serovar Hardjo. Cu Chi district was large and adjacent to many neighboring provinces such as Tay Ninh, Binh Duong and Long An, this leads to the transportation and trade of cattle occurred more frequently and created favorable conditions for pathogens to spread and infect easily (Tran, 2016).

Antibody titers in this study ranged from 1:100 to 1:800. With 270 *Leptospira*-positive serum samples, there were 145 (53.70%) positive cattle for agglutination test at 1/200 dilution and 11 cattle showed samples with an antibody titer against serovar L. hardjo bovis and L. hebdomatic at of 1/800 dilution (Table 5).

As mentioned above, samples are considered to be positive if agglutination occurred at a titer of 1/100 or more (OIE, 2014). However, a titer of ≥ 100 is often used as evidence of past exposure (Faine & WHO, 1982). According to the Centers for Disease Control and Prevention (CDCP, 1997), a titer of ≥ 200 is used to define a probable case with a clinically compatible illness and this defining case occurred in a population in which exposure to leptospirosis is uncommon; but, a higher cut-off titer is necessary for defining probable cases of leptospirosis in most tropical countries. According to the guidelines of the Veterinary Department of Ho Chi Minh City, cattle

infected with *Leptospira* spp. at antibody titer rates of 1/200 or higher must be treated with antibiotics. All cases of positive cattle at 1/200 to 1/800 titer should be isolated from their herds and treated with antibiotics. After this, the second serum samples would be collected at an interval of 10 to 14 days between samples (repeated sampling) to check if treatment was effective or infected cattle should be culled. In areas where leptospirosis is endemic, a single titer of $\geq$ 800 in symptomatic patients is generally indicative of leptospirosis (Faine, 1988).

4. Conclusions

In conclusion, of 446 cattle serum samples assayed for anti-leptospiral antibodies by microscopic agglutination testing, 85 (31.37%) reacted with serovar Hardjo hardjo bovis and 82 (30.26%) with serovar Hebdomadis. Results obtained in this study raised our concerns about the spread of infection of leptospirosis in cattle in Cu Chi district and will be used for the development of leptospirosis control program in Cu Chi district.

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Accumulation and distribution of heavy metal cadmium in sweet sorghum

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ABSTRACT

Many species of plants have been studied, as well as applied for cleansing the environment. Previous research has concluded that sorghum plants are highly tolerant to metal pollution and capable of reaching high biomass values in the presence of metals. However, the distribution of heavy metals in plant's parts has not been adequately studied. In this study, two varieties of sweet sorghum (Keller and E-Tian) were grown with 5 levels (0, 5, 10, 25 and 50 ppm) of cadmium (Cd) in order to investigate the accumulation of Cd in plant parts at the hard dough stage. The results clearly showed the absence of Cd in the seeds of the above plants. There was the presence of Cd at the second and fifth leaf when the level of Cd reached 25 - 50 ppm. There was a great correlation coefficient between Cd and the position of the internodes, namely 0.86, 0.96, 0.99, 0.98 with KE, and 0.86, 0.92, 0.94, 0.94 with ET at 5, 10, 25 and 50 ppm Cd ($P < 0.01$), respectively. The greater the internodes, the lower the accumulation of Cd. The aforementioned plants recorded the high accumulation of Cd in their roots, peaking at 23.27 µg/g (dried weight, dw) in Keller and 21.69 µg/g in E-Tian. Based on these results, it is concluded that the distribution of Cd in the studied sweet sorghum can be arranged in the following order: > stem > old leaves > young leaves.

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1. Introduction

Heavy metal contamination in soil has become a public concern due to industrial development and human activities, such as mining and smelting of metalliferous ores, electroplating, fertilizer and pesticide application, and fuel production (Garbisu & Alkorta, 2003). Excessive heavy metals, for example, cadmium (Cd), copper (Cu), lead (Pb), chromium (Cr), zinc (Zn), and nickel (Ni), in agricultural areas seriously threaten food safety and public health (Järup, 2003). Cadmium (Cd) has been placed at seventh rank among the top toxins, although Cd is a non-essential element for crop plants, it is easily taken up by plants growing on Cd-supplemented or Cd-contaminated soils, entering the food chain and causing damage to plant and human health (Rahat et al., 2012). Elimination or remediation of heavy metal contamination in soil is urgently needed to prevent humans and animals from toxicity.

Sorghum (*Sorghum bicolor* L.) is a pro-poor multipurpose crop providing food, feed, fiber, and fuel across a range of agro-ecosystems (Zheng et al., 2011). Sweet sorghum consists of natural variant cultivars of sorghum with abundant sucrose storage in culm and great biomass and is thereby considered an ideal feedstock for biofuel production (Kokyo et al., 2015). Sweet sorghum will be a competitive candidate species for soil remediation due to its great biomass and strong resistance to adverse environmental conditions.

To preliminarily evaluate its potential for phytoremediation, several morphological and physiological characteristics of sorghum were investi-

gated under heavy metal stresses (Cd, Pb, Zn, Cu) in previous studies (Zhuang et al., 2009; Liu et al., 2011; Soudek et al., 2013). There were several pieces of research which focus on the improvement ability of absorption heavy metal from the contaminated soil (Zhuang et al., 2009; Soudek et al., 2014; Ziarati et al., 2015). The aim of this study was to determine the absorption and distribution of Cd in sweet sorghum plant organs and its distribution in different organs of sweet sorghum.

2. Materials and Methods

2.1. Plant material and experimental design

The elite line of sweet sorghum Keller (KE) and E-Tian (ET) were chosen as plant materials. Keller (GRIN access code PI 653617) is an elite sweet sorghum line developed by DM Broadhead at US Sugar Crops Field Station at Meridan, Mississippi in 1982. E-Tian (literally meaning Russian Sweet in Chinese) was introduced into China in the early 1970s and known for having high Brix content in its stem (Zheng et al., 2011).

Soil was amended with $CdCl_2$ at final concentrations of 0, 5, 10, 25, 50 mg/kg. The group not treated with $CdCl_2$ was the control group. The soil was fertilized with base fertilizers (urea, diammonium phosphate, and potassium sulfate), following the technical process for high-yield land application.

Seeds were soaked in warm water at 28°C, then placed on a moist filter paper tray in a warm place for germination. After 3 days, the seedlings were subsequently transplanted into plastic pots (diameter: 30 cm; height 25 cm) with peat soil (2 kg soil for 2 seedlings per pot) and cultivated under glasshouse conditions (28 - 32°C with 14 - 16 h light/22 – 26°C with 8 - 10 h dark). The same care conditions and procedures were used for all experimental and control plants. Each experiment formula and control formula consisted of 12 plants with 3 replications. Leaves and internodes were numbered from the top to the bottom of the plant. The plant materials (root, internodes, leaves, and seed) were harvested when the oldest plants were in the hard dough stage.

2.2. Cd concentration assay

The plant samples were dried in a ventilated oven at 105°C for 30 min and 70°C for 48 h and subsequently ground into powders. 0.1 g of the ground sample was soaked in a mixture of HNO_3 and $HClO_4$ (3:1; v/v) according to Sun et al. (2008). Cd concentration was determined using a flame atomic absorption spectrometry Hitachi Z5000 (Tokyo, Japan).

2.3. Data analysis

The data were calculated using Statistix (version 10.0). Significant differences were determined by the least significant differences (LSD) at a 5% level of probability.

3. Results

3.1. Cd concentrations in leaves and seeds of sorghum

In the control treatment, the concentrations of Cd were not found in any organs of the plant such as the leaf, stem, root, or seed (Figure 1, 2, & 4; Table 1). For the treated plant, there was a significant difference in Cd accumulation in leaf among different Cd treatment levels. In the KE plant, Cd was absent in the second leaf at the lower concentration (5 and 10 ppm), and present when concentration was higher (25 and 50 ppm). The fifth leaf was observed with a presence of Cd at 5 ppm treatment. The highest Cd accumulation was recorded by treated 50 ppm Cd (0.9633 µg/g DW).

The results displayed the absence of Cd in the seed of a plant in both cultivars, even though Cd concentration was increased from 5 ppm to 50 ppm (Figure 1; Table 1). This result indicated that the transport of Cd from the root to the shoots and then to the seed was strongly inhibited. It also suggests that sweet sorghum can be used in safety for providing food, feed, and phytoremediation.

ET plants had a similar trend with KE plant for the accumulation of Cd in organs. By the lower Cd concentration treatments (5 and 10 ppm), Cd was completely absent in leaves and seeds. By the higher Cd treatments (25 and 50 ppm), the presence of Cd in the second leaf and fifth leaf was observed. The fifth leaf had a higher Cd concentration than the second leaf. The higher the concentration Cd treatment, the higher the concentration Cd accumulated in the leaf. There was no presence of Cd in the seed even though Cd concentration was increased from 5 to

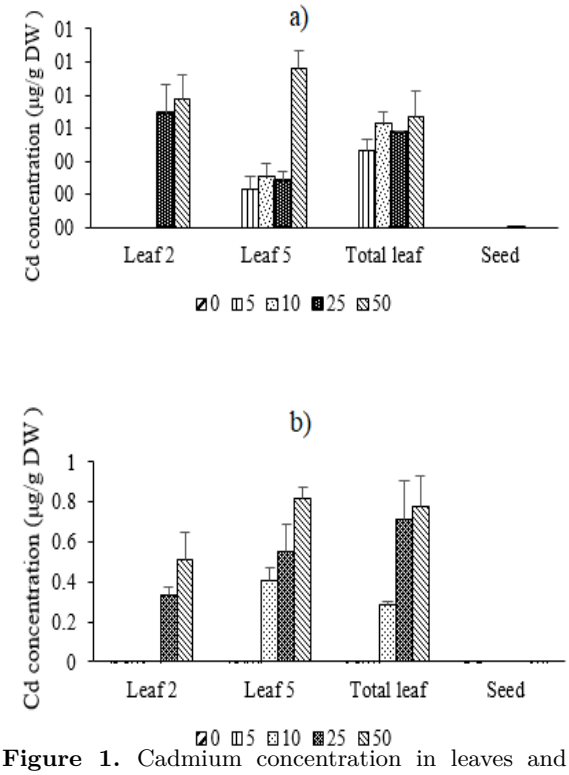

Figure 1. Cadmium concentration in leaves and seeds of a) sweet sorghum KE and b) ET. (DW: dried weight).

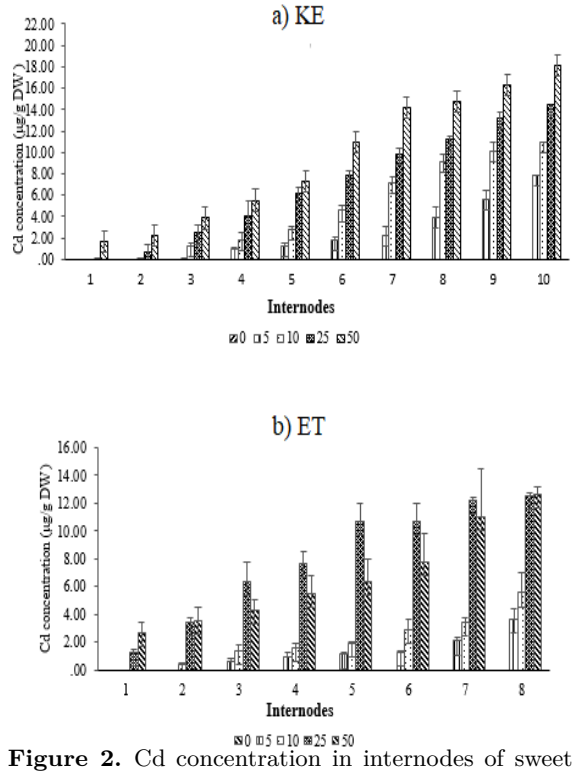

Figure 2. Cd concentration in internodes of sweet sorghum. The internodes were numbered according to the proximity to panicles. (DW: dry weight).

50 ppm, similar to the KE seed (Figure 1b, Table 1).

3.2. Cd concentrations in stems of sweet sorghum

Compared to the control, more Cd was significantly enriched in the stem of both sweet sorghum cultivars under excessive Cd condition (Figure 2). The accumulation and distribution of Cd in the internodes of sorghum stem were very different. There was a significant difference in Cd concentration between internodes in stem and between Cd treatment levels. This displayed the difference in the ability of absorption and accumulation Cd of sweet sorghum. The Cd concentration in the stem displayed more fold higher than Cd in leaf in both cultivars.

For the control plants, Cd was completely absent in the internodes of the stems of both cultivars. In KE treated Cd plants, under the lower 5 ppm Cd, Cd was not detected in the internodes 1st, 2nd, and 3rd. Cd was detected from the 4th internodes to the 10th internodes. The lower internode had higher Cd concentration (ranged from 0.92 µg/g DW to 7.81 µg/g DW at the 4th to 10th internode respectively) (Figure 2a). At the 10 ppm of Cd treatment, Cd was absent in the 1st, 2nd internode, and was detected from the 3rd to the 10th internodes. The highest Cd concentration was observed at the bottom internode of the stem (10th internode, Cd reached up 10.96 µg/g DW). Cd was recorded at the 2nd internode with 25 ppm Cd, Cd concentration in internodes was increased along the stem. The highest Cd at the 10th internode was 14.51 µg/g DW by 50 ppm Cd. By the highest 50 ppm Cd treatment, Cd was present at the 1st internode (Figure 2a; Table 1) and ranged from 1.65 to 18.13 µg/g DW at 1st to 10th internode respectively.

The similar trend was observed in ET, there was a significant difference in accumulation and distribution of Cd in stem among Cd treatment levels. At the lowest Cd treated plant (5 ppm), Cd in 1st and 2nd internode could not be detected. An increase in Cd was recorded from 3rd to the 8th internode (0.598 to 3.617 µg/g DW). At the Cd 10 ppm, Cd was absent in the 1st internode and present from 2nd to 8th internode (0.432 to 5.563 µg/g DW). At 25 and 50 ppm Cd, Cd accu-

Table 1. Cd concentration in organs of sweet sorghum at the hard dough stage (µg/g DW)

SS	Cd	Leaf 2	Leaf 5	Total leaf	Seed	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	Root
KE	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	5	ND	0.23 ± 0.07^{b}	0.47 ± 0.07^{b}	ND	ND	ND	ND	0.92 ± 0.29^{d}	1.29 ± 0.18^{c}	1.77 ± 0.29^{d}	2.27 ± 0.34^{d}	3.98 ± 0.76^{d}	5.63 ± 0.92^{d}	7.81 ± 0.86^{d}	2.85 ± 0.6^{d}
	10	ND	0.31 ± 0.08^{b}	0.63 ± 0.07^{ab}	ND	ND	ND	1.23 ± 0.37^{c}	1.86 ± 0.35^{c}	2.83 ± 0.69^{b}	4.56 ± 0.28^{c}	7.16 ± 0.49^{c}	9.19 ± 0.59^{c}	10.11 ± 0.69^{c}	10.96 ± 0.86^{c}	6.83 ± 0.2^{c}
	25	0.69 ± 0.18^{a}	0.28 ± 0.06^{b}	0.58 ± 0.01^{ab}	ND	ND	0.68 ± 0.2^{b}	2.48 ± 0.67^{b}	4.01 ± 0.71^{b}	6.15 ± 1.40^{a}	7.86 ± 0.53^{b}	9.80 ± 0.46^{b}	11.17 ± 0.59^{b}	13.22 ± 0.37^{b}	14.51 ± 0.55^{b}	13.93 ± 1.19^{b}
	50	0.77 ± 0.15^{a}	0.96 ± 0.11^{a}	0.67 ± 0.15^{a}	ND	1.65 ± 0.24	2.24 ± 0.65^{a}	3.88 ± 0.96^{a}	5.54 ± 0.67^{a}	7.25 ± 1.11^{a}	10.97 ± 0.54^{a}	14.14 ± 0.72^{a}	14.76 ± 0.75^{a}	16.26 ± 1.06^{a}	18.13 ± 0.96^{a}	23.27 ± 0.13^{a}
ET	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	ND
	5	ND	ND	ND	ND	ND	ND	0.59 ± 0.19^{c}	0.94 ± 0.30^{c}	1.10 ± 0.21^{c}	1.29 ± 0.12^{c}	2.08 ± 0.28^{bc}	3.62 ± 0.78^{c}	NA	NA	3.52 ± 0.29^{d}
	10	ND	0.41 ± 0.07^{c}	0.28 ± 0.02^{b}	ND	ND	0.43 ± 0.08^{b}	1.40 ± 0.36^{c}	1.56 ± 0.35^{c}	1.91 ± 0.13^{c}	2.89 ± 0.80^{c}	3.44 ± 0.27^{b}	5.56 ± 1.46^{b}	NA	NA	7.97 ± 0.49^{c}
	25	0.33 ± 0.05^{a}	0.55 ± 0.14^{b}	0.71 ± 0.19^{a}	ND	1.29 ± 0.16^{b}	3.47 ± 0.30^{a}	6.36 ± 1.44^{a}	7.64 ± 0.92^{a}	10.69 ± 1.33^{a}	10.67 ± 1.30^{a}	12.19 ± 0.18^{a}	12.56 ± 0.19^{a}	NA	NA	14.46 ± 0.42^{b}
	50	0.51 ± 0.14^{a}	0,.82 ± 0.06^{a}	0.77 ± 0.15^{a}	ND	2.64 ± 0.81^{a}	3.54 ± 0.96^{a}	4.28 ± 0.75^{b}	5.52 ± 1.26^{b}	6.36 ± 1.56^{b}	7.80 ± 1.97^{b}	11.00 ± 3.45^{a}	12.65 ± 0.56^{a}	NA	NA	21.69 ± 0.59^{a}

[a-d]Data with different letters in the same column of 1 cultivar means significant difference at 0.05 level. SS: sweet sorghum; I: internode; ND: not detected; NA: not applicable.

mulation was strongly increased along the stem. Cd accumulation in the 8th internodes was nearly 6-fold higher than that in the 1st internodes (Figure 2b; Table 1). Comparisons with the seedling stage showed Cd accumulation in the stem at the hard dough stage was observed 4 fold higher. This result indicates that the accumulation of Cd was increased more during the longtime of growth.

Under Cd exposure, the enriched Cd inhibited differential distribution within the stem of both KE and ET cultivars, which positively correlates with the position of internodes numbered according to the proximity to panicles. Increases in Cd concentration along the stem from the top internode to the lower internodes could be easily observed. There was a strong positive correlation between Cd concentration and internode positions along the stem.

The correlation coefficient of KE plant (0.86, 0.96, 0.99, 0.98 for KE and 0.86, 0.92, 0.94, 0.94 for ET by the treated 5, 10, 25 and 50 ppm Cd treatment respectively, $P < 0.01$). Cd preferentially accumulated in the lower internodes, while accumulating less in the upper ones (Figure 3). This indicates that the transport process of Cd from the root up to the tops was strongly inhibited. Hence, Cd concentration in the top internodes was very low, as in the leaf, and completely absent in the seed.

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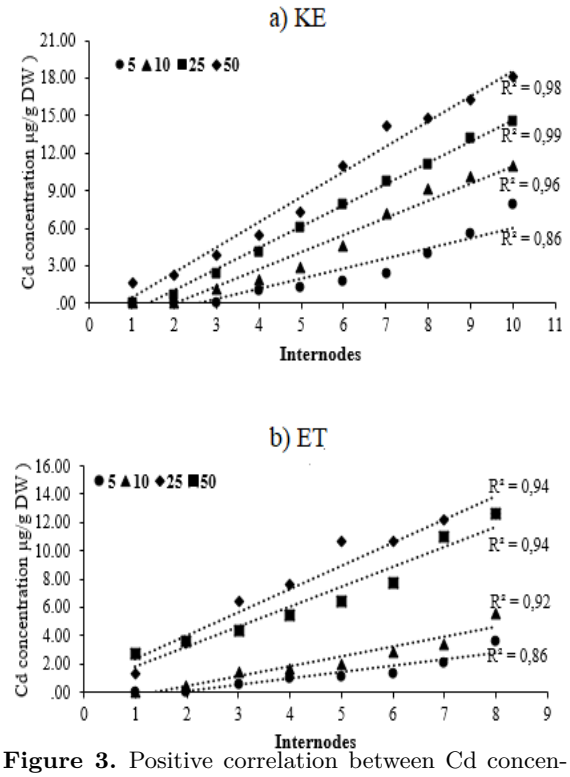

Figure 3. Positive correlation between Cd concentration and internode position along the stem. The internodes were numbered according to the proximity to panicles. R indicates the Pearson correlation coefficient.

3.3. Cd concentration in the root of sweet sorghum

KE and ET plants could accumulate a high concentration of Cd in the root. There was a significant difference among Cd exposed levels, which displayed differences in absorption and accumulation capacities of Cd in the plant (Figure 4).

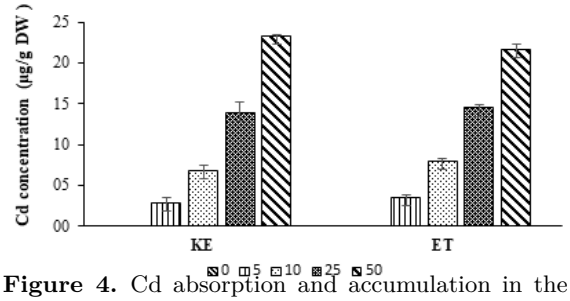

Figure 4. Cd absorption and accumulation in the root of sweet sorghum (DW: dry weight).

4. Discussion

The partitioning of Cd to different plant organs plays important role in the toxicity of Cd to plants. At the seedling and the hard dough stage, the distribution of Cd was different among organs of sweet sorghum. Results were consistent with previous studies, which showed was Cd in order root > stem > leaf (Barros et al., 2009; Soudek et al., 2013; Ziarati et al., 2015). Tuerxun et al. (2013) found that the Cd concentration in leaves, root, and stem of two sweet sorghum varieties increased as to the increased of added Cd content and to the elongation of exposure time. For both varieties of sweet sorghum, roots contained the highest Cd content, followed by stem and leaf (Tuerxun et al., 2013). However, Izadiyar & Yargholi (2010) studied on Cadmium absorption and accumulation in sorghum found that the maximum concentration can be observed in Sorghum root and the minimum concentration in sorghum stem. Cadmium concentration in different parts of the tested plant species is the following order of ranking: root > leaf > stem (Izadiyar & Yargholi, 2010). Probably, the response of sweet sorghum to Cd toxicity is not the same as other sorghums.

The results also displayed that the old leaf (the fifth leaf) can accumulate higher Cd than the young leaf (the second leaf) (Figure 1). Maria et al. (2013) indicated that roots and old leaves are the main metal sinks suggesting a defense or tolerance mechanism of the plants to avoid toxic levels in physiologically most active apical tissues (Maria et al., 2013). Moreover, the position of the fifth leaf was lower than the second leaf along the stem. Combined with the results about distribution Cd in the internodes of the stem (Figure 2), it could be concluded that the process of Cd transport in stem decided the distribution of Cd in aerial parts such as leaf, stem, and seed. Several studies determined the accumulation of Cd in the grain of sorghum (Zhuang et al., 2009; Angelova et al., 2011). Angelova et al (2011) studied heavy metals accumulated in different sorghums, included grain sorghum, technical sorghum, sugar sorghum, and Sudan grass grown on the soils contaminated with heavy metals (Pb, Cu, Zn, Cd). Their results showed that heavy metal content in the grains of Sudan grass, technical, and sugar sorghum were in the normal range (below the maximum permissible concentrations) and did not reach the phytotoxic levels (Angelova et al., 2011). In our result, although Cd treatment was increased from 5 ppm to 50 ppm, there was completely absent of Cd in seed in both cultivars of sweet sorghum (Figure 1; Table 1). Hence, in the present research, the accumulation of Cd was in the following order: roots > stems > old leaf > young leaf > seed. The accumulation of Cd in the stem of sweet sorghum was studied, but all previous studies have no attention to the distribution of Cd in each internode along the stem. This is also one of the new observations of our study.

The absorption and accumulation of Cd in the root of both sweet sorghum cultivars in this research were consistent with previous studies, root was the highest Cd accumulated part in the plant (Kokyo et al., 2015; Muratova et al., 2015; Nawab et al., 2015). Cadmium was accumulated primarily in the roots of sorghum plants and then transferred to the shoots. Sweet sorghum accumulated high Cd in roots and stems, while the shoots had a very low concentration of Cd. Because of the detoxification mechanism in the plant, the plant can uptake and accumulate Cd without being harmed (Cheng, 2003; Etim, 2012; Laghlimi et al., 2015).

The inhibition of transport of Cd from roots to shoots may reflect a self-defense mechanism. Studies of Pinto et al. (2006) showed that contamination levels of Cd resulted in a corresponding increase in concentrations of phytochelatin, produced by Sorghum. Phytochelatins are an important class of cysteine-rich poly peptides, the production of which was increased in response to excessive absorption of metal ions, such as Hg and Cd by plants (Pinto et al., 2006). Soudek et al. (2013) found that in the time dependence experiment the cadmium concentration in roots become generally greater than in shoots. The roots seem to have a barrier to prevent the transport of cadmium to shoots (Soudek et al., 2013).

Many species, including sweet sorghum, accumulate toxic metals mainly in the roots (Maria et al., 2013; Soudek et al., 2014; Ziarati et al., 2015). For sweet sorghum, increases in the concentrations of Cd in the soil lead to a higher accumulation of this metal in the root. Previous studies demonstrated that sorghum plants were highly tolerant to metal pollution and able to reach high biomass, even in the presence of heavy metals (Marchiol et al., 2007; Epelde et al., 2009; Liu et al., 2011). These results once again confirmed

the ability to clean up contaminated heavy metal Cd soil of sweet sorghum (Figure 4).

The amount of Cd accumulated in the plant is limited by several factors including 1) Cd bioavailability within the rhizosphere; 2) rates of Cd transport into roots via either the apoplastic or symplastic pathways; 3) the proportion of Cd fixed within roots as a Cd- phytochelatin complex and accumulated in the vacuole; and 4) rates of xylem loading and translocation of Cd (Rahat et al., 2012).

5. Conclusions

An overall increase of Cd concentration was found in all tissues of the plants (roots, stem, young, mature, and old leaves) by increasing the Cd contamination in the soil. Regardless of treatments, Cd concentration in roots always exceeded those in the aboveground dry matter because of a low translocation from roots to shoots. There were significant differences between the heavy metal contents in root, stem and leaf. The Cd was accumulated in the order that root > stem > old leaves > young leaves. The results clearly showed that the absence of Cd in the seeds of the above plants.

This study detected that sorghum also had considerable accumulation ability to Cd in root and stem. The absence of Cd in seed and inhibition of translocation Cd from root to the shoots may represent the avoid effect on the food chain, which should be suitable for bioremediation.

Furthermore, Cd is accumulated preferentially in the lower internodes while scarcely accumulated in the upper internodes of both sweet sorghum lines KE and ET. These results suggested that excessive Cd accumulation is avoided in leaves, inflorescence, and seeds essential for photosynthate fixation and reproduction. Therefore, Cd accumulation in lower internodes benefits the resistance of sweet sorghum to Cd toxicity.

In conclusion, sweet sorghum should be a competitive candidate species for soil remediation due to its great biomass and strong resistance to adverse environmental conditions.

Conflicts of interest

The authors declare no conflicts of interest.

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Optimization of aqueous extraction conditions for bioactive compounds from fresh *Pouzolzia zeylanica* plant using response surface methodology

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ABSTRACT

Response surface methodology was applied to optimize the extraction of phenolic compounds from fresh *Pouzolzia zeylanica* plant using hot water as a solvent. A central composite design (CCD) in form (2^3+star) was used to investigate the effects of two independent variables, namely, extraction temperature (70 to 90°C) and extraction time (20 to 40 min). The dependent variables were the content of anthocyanin, flavonoid, polyphenol, tannin and total soluble solids of extracted solution. A second-order polynomial model was used for predicting the response. The results showed that the optimal extraction process was obtained at 84.4°C for 31.7 min. The experimental values agreed with predicted within a 95% confidence interval. Consequently, the contents of anthocyanin, flavonoid, polyphenol and tannin were 38.66 mgCE/100 g, 3.01 mgQE/g, 5.17 mgGAE/g, 4.07 mgTAE/g fresh weight, and total soluble solids content was 0.73%, respectively.

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1. Introduction

Pouzolzia zeylanica is a medicinal source that people of Asia countries have used to treat various kinds of diseases by traditional methods. In Vietnam, this plant was popularly cultivated in the Mekong Delta region, it can be used as fresh or dried plant, decoction drunk to treat cough, pulmonary tuberculosis, sore throat, enteritis and dysentery (Vo, 2012). Several *in vitro* researches have indicated ethanolic extracts of *Pouzolzia zeylanica* possessed antibacterial, antifungal and cytotoxic activities (Saha et al., 2012; Sara & Paul, 2012); it had no oral acute toxicity at the oral dose of 10 g material powder/kg (Tran et al., 2010). Traditionally, this plant was prepared as an infusion with water, to make tea.

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures (Handa et al., 2008). The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular. The obtained crude extracts contain a complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids. Some of the initially obtained extracts may be ready for use as medicinal agents or beverages but some need fur-

ther processing.

In addition, we have known since decades that chemical constituents as an extractable matter which obtained from the extraction process were influenced by extraction parameters, also influenced by the quality of the medicinal plant (Vyas et al., 2013). So, if the extraction process can be optimized in terms of bioactive compounds content such as anthocyanin, flavonoid, polyphenol and tannin. They could have had potential as beverages or concentrated products with medicinal properties. The presence of phenolic compounds in the extracted solution had effect on biological value of the final product . Therefore, it is necessary to determine the effects of extraction time and temperature on the content of phenolic compounds.

2. Materials and Methods

2.1. Chemicals and reagents

Folin-Ciocalteu, Folin-Denis reagents and quercetin, gallic acid, tannic acid were obtained from Sigma Chemical Co. (USA) and Merck Chemical Supplies (Germany). All the chemicals, including the solvents, were of analytical grade.

2.2. Sample preparation and extraction

Pouzolzia zeylanica plants were collected in April 2017 from a household in Hoa Binh village, Cho Moi district, An Giang province with 20-30 cm height. It was cleaned with tap-water, cut into small pieces about 2-3 cm long. After that, the samples of *Pouzolzia zeylanica* were extracted with water using an airtight extractor (model GPA CC1-181907, DidatecTechnologie France, 2007). Stirring rate was maintained at 90 (rpm). The extract samples were fixed a volume of 5 liters and solution to the material ratio of 15:1, v/w. The samples were extracted at temperature of (63, 70, 80, 90 and 97°C), in the duration of (13, 20, 30, 40 and 47 min). The extracts were filtered by cotton cloth and determined their volumes. Subsequently, the extracts were filtered using Buchner funnel with Whatman's No 1 filter paper. The crude extract was diluted at an appropriate ratio using for analysis.

2.3. Experimental design and statistical analysis

In this study, response surface methodology (RSM) with central composite design (CCD) in form (2^3+star) was used to investigate the effects of two independent variables: X (extraction temperature) and Y (extraction time) on the extraction of anthocyanin, flavonoid, polyphenol and tannin contents. The independent variables were coded at five levels (-α, -1, 0, +1, +α) and the complete design consisted of 13 experimental points, including five replications of the center points (Table 1). The experimental design and statistical analysis were performed using Statgraphics plus 16.0 for Windows. A quadratic equation (second-order polynomial equation) was used to fit the results:

$Z = b_0 + b_1X + b_2Y + b_{1.2}XY + b_{1.1}X_2 + b_{2.2}Y_2$

Where Z is the predicted response parameter, bo is a constant, b1, b2, b1.1, b2.2 and b1.2 are the regression coefficients; X and Y are the levels of the independent variables (extraction temperature and time). Experimental data were then fitted to the selected regression model to achieve a proper understanding of the correlation between each factor and different responses. This correlation was obtained by estimating the numerical values of the model terms (regression coefficients), whose significance was statistically judged in accordance with t-statistic at a confidence interval of 95%. Non-significant ($P > 0.05$) terms were deleted from the initial equation and data were refitted to the selected model. This work helped that the models will have a higher correlation coefficient R. The compatibility of the mathematical models was fitted by RSM and evaluated by ANOVA, based on the F-test, the probability value (P) of lack-of-fit and on the percentage of total explained variance (R^2), and also on the adjusted determination coefficient (R^2_{adj}). These variances provide a measurement of the variability in the observed response values that could be explained by the experimental factors and their linear and quadratic interactions. Simultaneous optimization of the desirability function was performed in order to maximize the content of anthocyanin, flavonoid, polyphenol, tannin and soluble solids.

Table 1. Coded and uncoded experimental values of extraction temperature and time of fresh *Pouzolzia zeylanica* and results from the extract solution assays

Number Run	Independent variables		Responses			
	Temperature (°C)	Time (min)	Anthocyanin (mgCE/100 g)	Flavonoid (mgQE/g)	Polyphenol (mgGAE/g)	Tannin (mgTAE/g)	Soluble solids (%)
1	66 (-α)	30 (0)	30.15 ± 0.95	2.25 ± 0.17	3.98 ± 0.25	3.06 ± 0.19	0.54 ± 0.07
2	70 (-1)	20 (-1)	31.42 ± 0.86	2.19 ± 0.09	4.08 ± 0.16	3,09 ± 0.12	0.53 ± 0.04
3	70 (-1)	40 (+1)	35.61 ± 0.92	2.56 ± 0.12	4.66 ± 0.15	3.51 ± 0.22	0.58 ± 0.05
4	80 (0)	16 (-α)	33.91 ± 0.88	2.44 ± 0.15	4.71 ± 0.18	3.45 ± 0.11	0.57 ± 0.01
5	80 (0)	30 (0)	38.26 ± 0.65	2.97 ± 0.19	5.11 ± 0.21	3.98 ± 0.15	0.69 ± 0.06
6	80 (0)	30 (0)	37.85 ± 0.33	2.91 ± 0.11	4.98 ± 0.09	4.01 ± 0.07	0.72 ± 0.09
7	80 (0)	30 (0)	37.82 ± 0.11	3.01 ± 0.21	5.04 ± 0.22	3.86 ± 0.13	0.68 ± 0.02
8	80 (0)	30 (0)	38.89 ± 0.83	2.89 ± 0.18	5.07 ± 0.17	3.95 ± 0.25	0.73 ± 0.01
9	80 (0)	30 (0)	39.06 ± 0.76	2.95 ± 0.14	5.18 ± 0.19	3.88 ± 0.16	0.71 ± 0.08
10	80 (0)	44 (+α)	37.08 ± 0.57	2.86 ± 0.22	4.96 ± 0.08	4.01 ± 0.14	0.66 ± 0.06
11	90 (+1)	20 (-1)	38.02 ± 0.89	2.78 ± 0.05	5.08 ± 0.11	3.98 ± 0.08	0.65 ± 0.04
12	90 (+1)	40 (+1)	35.11 ± 0.94	2.85 ± 0.25	4.99 ± 0.13	4.02 ± 0.17	0.71 ± 0.03
13	94 (+α)	30 (0)	36.22 ± 0.81	2.81 ± 0.08	4.95 ± 0.14	4.01 ± 0.15	0.72 ± 0.08

Data presented as mean (n = 3) ± SD (Standard Deviation).

2.4. Determination of chemical composition of *Pouzolzia zeylanica* L. Benn

2.4.1. Total anthocyanin content (mgCE/100 g FW)

The determination of monomeric anthocyanin was conducted by pH-differential method (Ahmed et al., 2013). The samples perform dilutions in 50 mL volumetric flasks. The volumetric pipets are used for addition of the test portion. The maximum test portion added should be $\leq$ 10 mL (the ratio of test/buffer is 1/4, v/v) and not to exceed the buffer capacity of the reagents. The absorbance of test portion diluted with pH 1.0 buffer and pH 4.5 buffer is determined at both 520 nm and 700 nm. Total monomeric anthocyanins were expressed as cyanidin-3-glucoside. Sample absorbance was read against a blank cell containing distilled water. The absorbance (A) of the sample was then calculated according to the following formula:

$A = (A_{520} - A_{700})pH\ 1.0 - (A_{520} - A_{700})pH\ 4.5$

Total anthocyanin content (TAC) in the sample was calculated according to the following formula:

TAC (mgCE/100 g) = (A x MW x DF x V x 1000)/(ϵ x 1 x W)

Where DF is dillution factor, MW is cyanidin-3-glucoside molecular weight (449,2), ϵ is molar absorptivity (26,900), V is volume of the obtained extracts, in litre, 10^3 is factor for conversion from g to mg, W is the weight of material sample, in gram.

2.4.2. Total flavonoid content (mg QE/g FW)

Aluminum chloride colorimetric method was used for flavonoids determination (Eswari et al., 2013). About 1 mL of the crude extracts/standard of different concentrations was mixed with 3 mL ethanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M sodium acetate and 5.8 mL of distilled water. It remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with spectrophotometer against blank. The calibration curve was prepared by diluting quercetin in ethanol ($y = 0.0054x + 0.0026$ and $r^2 = 0.9995$). The total flavonoid content (TFC), milligrams of quercetin equivalents (QE) per gram fresh weight (FW), was calculated by the following formula:

TFC (mgQE/g) = [(A – 0.0026) x DF x V]/

(0.0054 x W)

Where A is the absorbance of the test samples; DF is the dilution factor; V is volume of the obtained extracts, in litre; W is the weight of material sample, in gram.

2.4.3. Total polyphenol content (mg GAE/g FW)

Total polyphenol content was determined by Folin-Ciocalteu reagent method (Hossain et al., 2013). Each crude extract (0.2 mL) was taken in a test tube and added 10% Folin-Ciocalteu reagent (1.5 mL). Then all test tubes were kept in a dark place for 5 min. Finally, 5% Na_2CO_3 (1.5 mL) was added to solution and mixed well in a vortex. Again, all the test tubes were kept in the dark for 2 h. The absorbance was measured for all solutions by using UV-spectrophotometer at constant wavelength 750 nm. Total polyphenol concentrations were quantified by a calibration curve obtained from measuring the absorbance of a known concentration of gallic acid standard in ethanol ($y = 0.0082x + 0.0595$ and $R^2 = 0.9996$). The total polyphenol content (TPC), milligrams of gallic acid equivalents (GAE) per gram fresh weight (FW), was calculated by the following formula:

TPC (mgGAE/g) = [(A – 0.0595) x DF xV]/(0.0082 x W)

Where A is the absorbance of the test samples; DF is the dilution factor; V is the volume of the obtained extracts, in litre; W is the weight of the material sample, in gram.

2.4.4. Tannin content (mg TAE/g FW)

Tannin content was determined by Folin-Denis method (Laitonjam et al., 2013). Each crude extract (0.5 mL) was taken in a test tube and added distilled water (0.5 mL). Finally, the samples were treated with 0.5 mL of freshly prepared Folin-Denis reagent and 20% sodium carbonate (2 mL) was added, shaken well, warmed on boiling water-bath for 1 minute and cooled to room temperature. The absorbance of the coloured complex was measured at 700 nm. Tannin concentration was quantified based on the calibration curve of tannic acid in ethanol ($y = 0.0098x + 0.0478$ and $R^2 = 0.9996$). The tannin content (TC), milligrams of tannic acid equivalents (TAE) per gram fresh weight (FW), was calculated by the following formula:

TC (mgTAE/g) = [(A – 0.0478) x DF x V]/(0.0098 x W)

Where A is the absorbance of the test samples; DF is the dilution factor; V is volume of the obtained extracts, in litre; W is the weight of the material sample, in gram.

2.5. Total soluble solids (%)

Determination total soluble dry matter content was conducted by following protocol of Giang et al. (2013). Take 30 mL extract solution to a dried cup that determined weight. The heating in boiled water until the evaporation of water was finished. Then, put it in oven at 100-105°C, drying until the weight of cup was constant. The content of total soluble solids (TSS) in extract solution was determined by the following formula:

TSS (%) = [(G_2 – G_1) x 100]/G

Where G is the weight of test solution, G_1 is weight of cup, G_2 is weight of cup and test solution.

3. Results and Discussion

The results from Table 1 showed that when the extraction temperature and time changed, the content of bioactive compounds and total soluble solids in the extracts varied accordingly: the anthocyanin content was in the range of 30.15÷39.06 mgCE/100 g; flavonoid 2.19÷3.01 mgQE/g; polyphenol 3.98÷5.18 mgGAE/g; tannin 3.06÷4.01 mgTAE/g FW (fresh weight); and total soluble solids was from 0.53÷0.73%.

Response surface and contour plots in Figure 1 showed the extraction temperature and time had effect on the content of bioactive compounds and soluble solids according to the second-order model with significant levels ($P < 0.05$). When extraction temperature and time increased, the content of bioactive compounds in the extracted solution had increasing trend, and achieved optimal value, then had a decrease. Specifically, the anthocyanin content increased and reached an optimal value of 38.72 mgCE/100 g at 83.7°C and 30.3 min (Figure 1a and 1a'); flavonoid achieved an optimum value of 3.01 mgQE/g at 84.4°C and 33.3 min (Figure 1b and 1b'); polyphenol reached an optimal value of 5.17 mgGAE/g at 85.6°C and 30.6 min (Figure 1c and 1c'); tannin reached an optimum value of 4.10 mgTAE/g at 87.7°C and 34.3 minutes (Figure 1d and 1d').

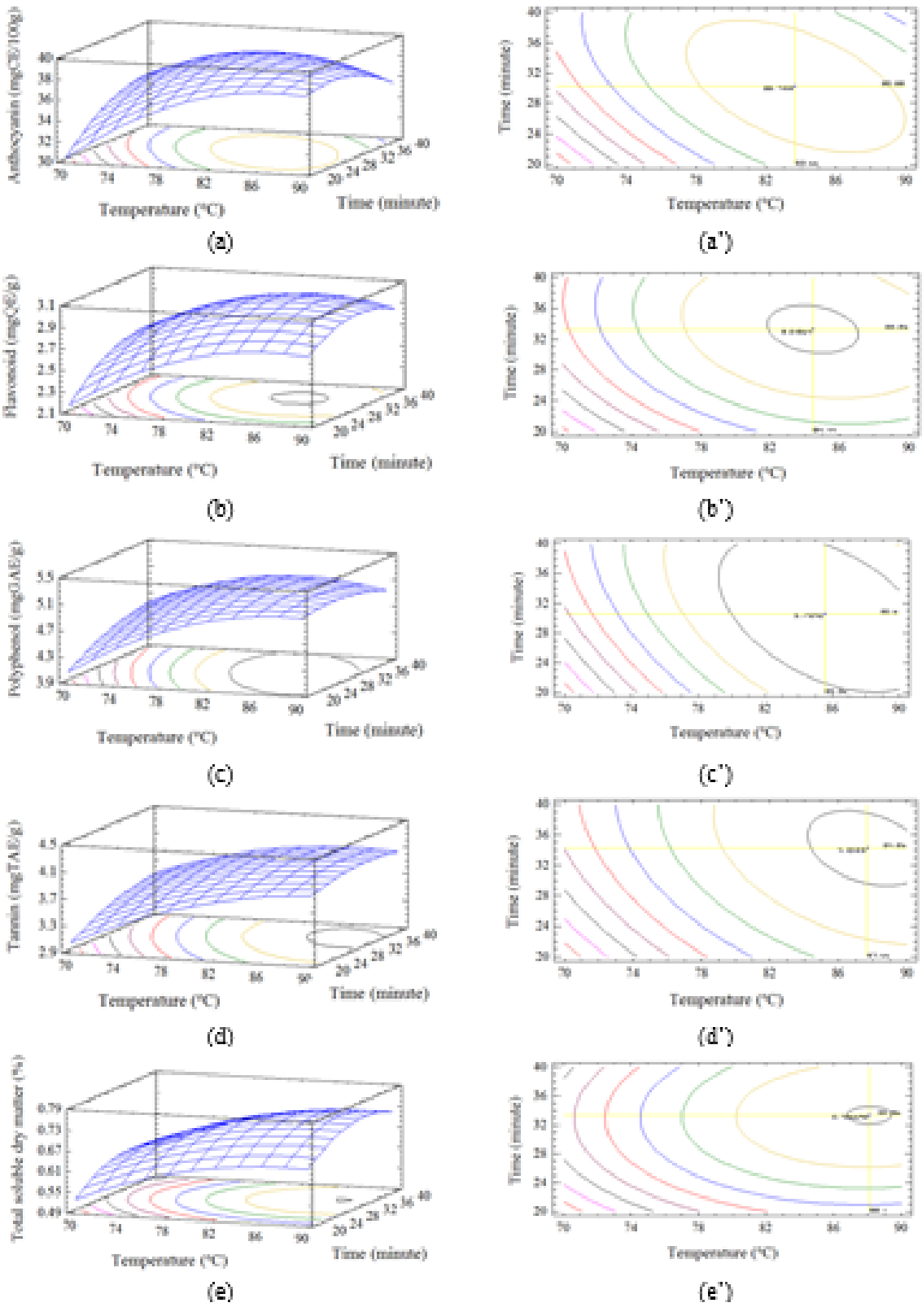

Figure 1. Response surface and contour plots for the content of anthocyanin (a, a'); flavonoid (b, b'); polyphenol (c, c'); tannin (d, d') and total soluble solids (e, e') in different temperature and time.

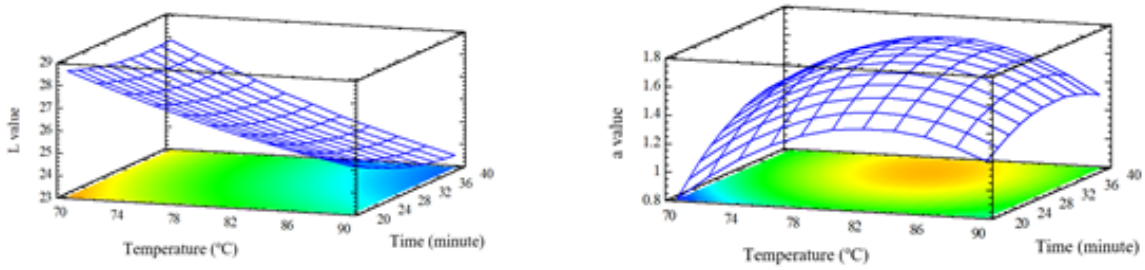

Figure 2. Response surface and contour plots for the color parameters of extract such as L value (a) and a value (b) in different temperature and time.

The results showed that the extraction of bioactive compounds with water solvent was carried out at high temperature (83÷87°C) and short extraction time in the range of 30÷34 minutes. Since most bioactive compounds were sensitive to high temperatures, long extraction time could lead to the decomposition of bioactive compounds (Vu & Ha, 2009). According to Rajha et al. (2014) extraction of phenolic compounds (polyphenols, flavonoids, tannins and anthocyanins) from grape skins found the optimum extraction parameters of 81°C and 140 min for non-grinding grape grains and 88°C for 5 min grape skins were crushed. Sheng et al. (2013) explained that bioactive compounds were better released from plant cells by reducing the viscosity of the solvent and increasing the molecular motion with increased temperature during extraction. The results of Vu & Ha (2009) showed that the polyphenol content increased when the extraction temperature was increased from 70÷90°C during the polyphenol extraction process from green tea. The increase of extraction temperature would increase the phenolics extraction efficiency reported by many authors (Spigno & Faveri, 2007; Spigno et al., 2007; Rajha et al., 2012). Whenever temperature was increased, it reduced surface tension and viscosity, improving the solubility of the solute (Ramos et al., 2002). However, if higher temperature could occur phenolic compounds decompose. The phenolic compounds could avoid composition as the short duration of the extraction process, but high temperatures and long time would have a negative effect on the polyphenol content, oxidation or decomposition could occur (Yilmaz & Toledo, 2006). Under the effect of oxidation-reduction enzymes, plant tannin was readily oxidized and condensed into colorful or colorless products that directly affected the color of the product (Le, 2003). The appropriate temperature for extraction of tannin from bark is between 90÷100°C (Connolly, 1993). Some authors had shown that the effect of temperature on flavonoid extraction, when the extraction temperature was higher than the optimum temperature, reduced the flavonoid content (Sheng et al., 2013).

Response surface and contour plots in Figures 1e and 1e' showed that the extraction temperature and time also influenced the second order model to the soluble solids content of the extract. Dissolved solids increase with increasing temperature and extraction time and achieved high values in the range of 82÷90°C, dissolved solids reached the optimum value of 0.74% at 88.1°C and 33.4 min. The heat treatment increased the solubility and diffusion of the compounds. The heating decreased the viscosity of the extracting solvent, but it increased the mass transfer and helps the solvent penetrates easily into the cell (Al-Farsi & Lee, 2008). On the other hand, according to Mohammad et al. (2011), high temperatures could reduce cellular barriers by weakening the walls and cell membranes, making the solvent more easily exposed to the compounds, increasing the ability to extract solutes into the extract solution.

The results in Figure 2a showed that the light-dark (L) value tended to decrease as the temperature and the extraction time was increased. The samples with the darkest color (L = 23.35) at the extraction temperature and time were 94°C and 30 min, respectively. The sample had the lightest color (L = 29.24) at 66°C and 33 min. Meanwhile, the results in Figure 2b showed that the green-red value (a) trended to increase when the extraction time was extended at low temperatures from 66÷80°C but when raised to 90÷94°C and extending the extraction time, a value trended to decrease. The highest red color (a = 1.97) was

extracted at 80°C for 44 min and the lowest red color (a = 0.89) at the temperature and extraction time of 66°C and 33 min. This could be explained by increased temperature or prolonged extraction time, which increased the ability to extract color compounds (phenolics compounds) in medicinal plants so that the L value would decrease (darker color) because L had value of 100÷0, the value of a would increase (the color would be redder) because a value had green value (-) and (+) is red. However, when the optimum condition was obtained, the phenolics would decompose (especially anthocyanin), reducing the red color of the extract.

In addition, the results of ANOVA statistical analysis of the data in Table 2 showed that the correlation model constructed with linear, interactive and quadratic coefficients of the temperature and time had effect on the anthocyanin, flavonoid, polyphenol, tannin and soluble solids content of the obtained extract with confident level of 95%. In which, the linearity coefficient of the temperature factor had significant effect on the anthocyanin compounds, flavonoid ($P < 0.001$), the time factor had a significant effect ($P < 0.01$); the coefficient of squared and interaction of temperature and time factors had effect in confident level ($P < 0.05$); except for the interaction coefficient of extraction temperature and time, there was no effect on soluble solids content ($P > 0.05$).

The good correlation model required a match between the actual and theoretical data, so the constructed model with Lack of fit test was not statistically significant (Zabeti et al, 2009). In addition, the correlation model should have a correlation coefficient of R^2 greater than 0.8 (Guan & Yao, 2008). The results in Table 2 showed that the correlation coefficient of the predicted models was $R^2 > 0.951$ and the P for lack of fit was $0.1379 > 0.05$. The model's suitability was very high and there was good compatibility between experimental and predictive data (Figure 3).

3.1. Multiple response optimization

Extraction was widely known as an extraction process of bioactive substances from plant materials. Several factors could contribute to the effects of bioactive compounds extracted, including the method of extraction, temperature and extraction time, rate of materials and solvent (Pinelo et al., 2005a & 2005b; Chew et al., 2011).

Table 2. Mathematical equations that describe the responses (anthocyanin, flavonoid, polyphenol, tannin, soluble solids) in response to temperature and time

Response variables	Regression Equations	R^2	R^2 (adjusted for d.f.)	P-value (lack-of-fit)
Anthocyanin (mgCE/100 g)	$Z = -189.075 + 4.6301X + 2.253Y - 0.0245X^2 - 0.018XY - 0.013Y^2$	0.951	0.916	0.1379
Flavonoid (mgQE/g)	$Z = -15.635 + 0.378X + 0.162Y - 0.002X^2 - 0.001XY - 0.002Y^2$	0.985	0.975	0.6371
Polyphenol (mgGAE/g)	$Z = -21.878 + 0.558X + 0.209Y - 0.003X^2 - 0.002XY - 0.001Y^2$	0.981	0.968	0.6553
Tannin (mgTAE/g)	$Z = -15.165 + 0.381X + 0.151Y - 0.002X^2 - 0.001XY - 0.001Y^2$	0.978	0.963	0.3997
Soluble solidss (%)	$Z = -2.792 + 0.069X + 0.029Y - 0.0004X^2 + 0.00003XY - 0.0005Y^2$	0.971	0.951	0.9810

X = Extraction temperature (°C); Y = Extraction time (min).

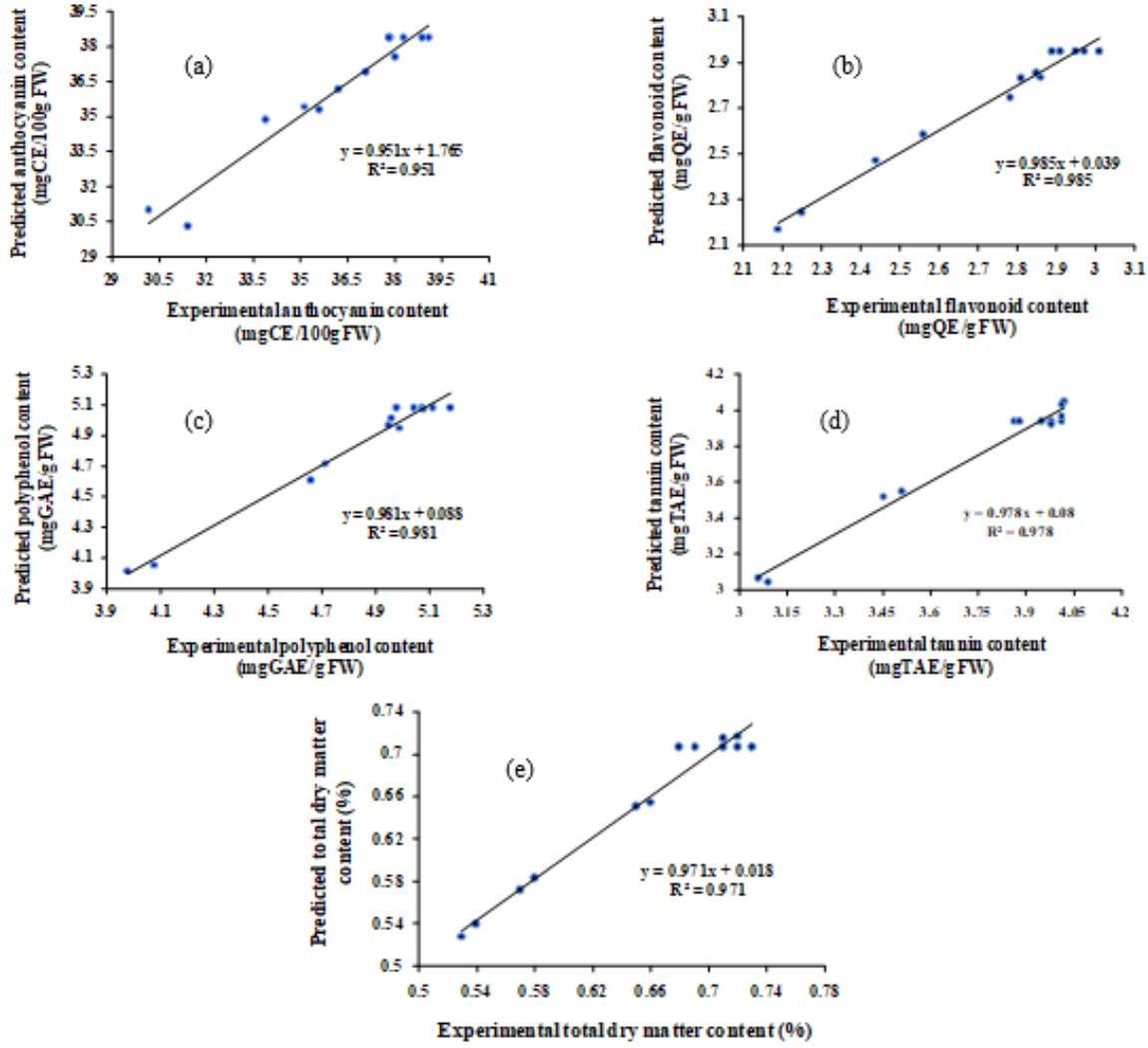

Figure 3. Correlation between the experimentally and the estimated values for anthocyanin (a), flavonoid (b), a polyphenol (c), tannin (d) and total soluble solids (e) using the models described in equation 1, 2, 3, 4, 5; respectively (as shown in Table 2).

The responses (anthocyanin, flavonoid, polyphenol, tannin and soluble solids content) were optimized separately, therefore allowing the targeting of a certain class of compounds only by varying the extraction parameters. Yet, the desirability function in the RSM was utilized to reveal the combination of the parameters (temperature and time) capable of simultaneously maximizing all the responses. The overplay plot (Figure 4) showed the outlines superposition of all the studied responses and the simultaneous optimum for all responses was showed by the black spot.

The optimum extraction parameters were obtained from the model with a temperature of 84.4°C and a time of 31.7 min. At this optimal extraction parameter, the content of the anthocyanin, flavonoid, polyphenol, tannin and dissolved solids was 38,66 mgCE/100 g; 3.01 mgQE/g; 5.17 mgGAE/g; 4.07 mgTAE/g fresh weight and 0.73%, respectively.

3.2. Test the predicted values from the model

To test the optimal values obtained from the predicted models, the study performed according to the best parameters found: extraction at 85°C for 32 min; then filtered and retrieved the extract and conduct analyzed to determine the

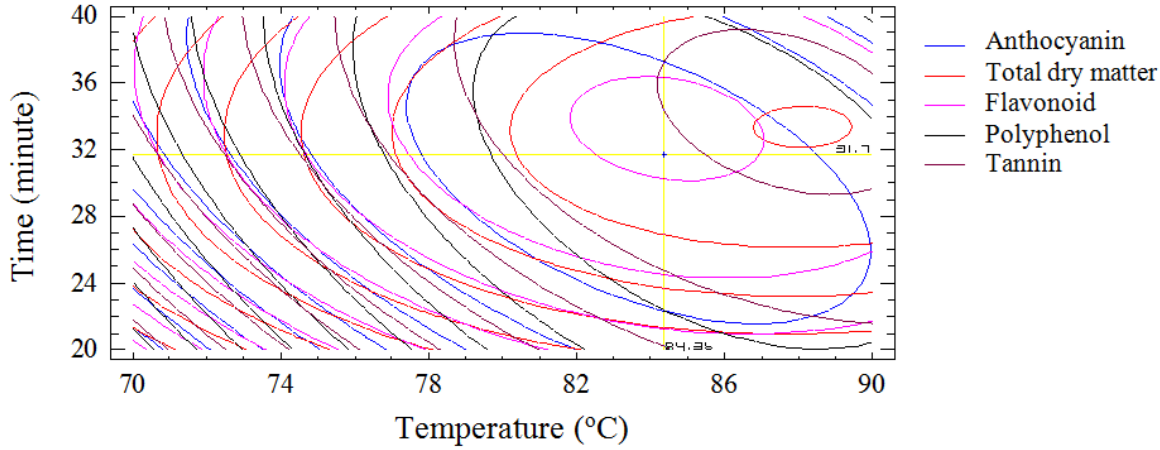

Figure 4. Superposition contour plots, showing the best experimental parameters that maximize bioactive compounds content and total dry matter of extract solution (the black spot shows the optimum for all the responses).

Table 3. Comparison of test values with calculated values of optimal models

No.	Analytical targets	Test value*	Calculated value	Differential percentage (%)
1	Anthocyanin (mgCE/100 g FW)	37.19 ± 0,97	38.66	3.80
2	Flavonoid (mgQE/g FW)	3.14 ± 0,07	3.01	4.14
3	Polyphenol (mgGAE/g FW)	5.25 ± 0,19	5.17	1.52
4	Tannin (mgTAE/g FW)	3.94 ± 0,15	4.07	3.19
5	Soluble solids (%)	0.71 ± 0,01	0.73	2.74

(*) Mean value (n=3) and ± SD (Standard Deviation).

content of bioactive compounds and dissolved solids. The content of anthocyanin, tannin and dissolved solids were lower than predictive values by 3.80%; 3.19% and 2.74%. Meanwhile, the levels of flavonoid and polyphenol were higher than predictive values by 4.14% and 1.52% respectively (Table 3). The difference was within the allowable limit (< 5%). The result of this difference was that the optimum extraction conditions of the compounds found in the model were between 83.7÷88.1°C and 30.3÷34.3 minutes.

4. Conclusions

Response Surface Methodology (RSM) is a highly reliable method in predicting optimizing models. Using RSM to find the most suitable temperature and time to extract bioactive compounds and soluble solids at the same time could minimize the degradation of these bioactive substances. Therefore it could improve the quality of compounds after the extraction. The extraction temperature and time were 85°C and 32 min. At this condition, the content of anthocyanin, flavonoid, polyphenol, tannin and soluble solids were 37.19 mgCE/100 g; 3.14 mgQE/g; 5.25 mgGAE/g; 3.94 mgTAE/g fresh weight, 0.71%, respectively. This method could become an alternative technique to apply in solid-liquid extraction the bioactive compounds in *Pouzolzia zeylanica* at the industrial scale.

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