UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF ZENK AND FOXP1 GENE IN A BRAIN OF A PARROT

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EXPRESSION OF ZENK AND FOXP1 GENE IN A BRAIN OF A PARROT

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It is hereby certified that we have read this project paper entitled “Expression of ZENK AND FOXP1 gene in a parrot”, by Nurul Afiqah binti Yazid and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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DEDICATION

I dedicate this thesis with love and appreciation to :

My parents

Yazid bin Habib

Azizah binti Omar

My siblings

Mohd Azrul bin Yazid & family

Nurulafzan binti Yazid & family

My Supervisor

Dr. Hafandi bin Ahmad

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

EKSPRESI GEN ZENK DAN FOXP1 DI DALAM OTAK BURUNG NURI

Oleh

Nurul Afiqah Yazid

2015

Penyelia : Dr. Hafandi Ahmad

Penyelia Bersama : Prof. Madya Dr. Jalila Abu

Burung nuri adalah salah satu burung yang terkenal dengan kemahiran komunikasi dan vokal mereka . Berdasarkan kajian yang dilakukan sebelum ini, pengeluaran vokal dikawal oleh otak depan dan juga ianya berkaitan dengan perubahan beberapa gen ungkapan yang bertanggungjawab mengawal persepsi lagu dan pengeluaran bunyi. Walaupun banyak kajian yang dilaporkan berkenaan ekspresi gen yang dikaitkan dengan perkembangan pembelajaran dan ingatan pada manusia penemuan terhad dilaporkan pada gen yang berkaitan dengan penghasilan vokal oleh burung nuri. ZENK dan FOXP1 adalah gen yang bertanggungjawab dalam kemahiran komunikasi dan vokal burung nuri. Oleh itu , kajian ini dijalankan untuk menganalisis ekspresi gen yang dikaitkan dengan pengeluaran vokal yang dikawal oleh ZENK dan FOXP1. Tisu otak diambil daripada burung yang telah mati dan disimpan di dalam RNA *later*. Pengekstrakan RNA dilakukan diikuti oleh qPCR untuk kuantifikasi gen FOXP1 dan ZENK dalam sampel. Gel elektroforesis telah dilakukan untuk membuktikan bahawa terdapat kehadiran FOXP1 dan ZENK dalam otak nuri . Kepekatan RNA yang berbeza telah digunakan iaitu 170 ng/ml, 17 ng/ml, 1.7 ng/ml, 0.17 ng/ml dan ng 0.02/ml. Hasilnya telah didokumenkan sebagai nilai ambang kitaran (CT). Dalam kepekatan RNA 170 ng/ml, nilai CT terendah dicatatkan iaitu 22.91 dalam ZENK dan 23.39 dalam FoxP1 manakala kepekatan RNA 0.02 ng/ml mempunyai nilai CT 23.94 untuk ZENK dan 23.99 untuk FOXP1 . Oleh kerana nilai CT adalah berkadar langsung kepada ungkapan gen , kita dapat membuat kesimpulan bahawa gen adalah kehadiran dalam jumlah yang besar dalam kepekatan yang tinggi RNA .

Kata kunci : burung nuri, ekspresi gen, FOXP1, ZENK, PCR

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Project.

EXPRESSION OF ZENK AND FOXP1 GENES IN A PARROT BRAIN

By

Nurul Afiqah Yazid

2015

Supervisor : Dr Hafandi Ahmad

Co-supervisor : Assoc. Prof. Dr. Jalila bt Abu

Parrots are one of the songbirds that are well known for their vocalization skills. Based on previous study, the production of songbirds is being controlled by the forebrain and also related with changes of some genes expression that is responsible for song perception and production. Although many studies on the genes expression associated with the learning and memory development in humans are being reported, limited findings are reported on genes related with the songbird. The ZENK and FOXP1 are the most likely genes that are responsible in the songbird. Therefore, we conducted a study to analyze the gene expression associated with songbirds which are ZENK and FOXP1. A brain tissue of dead parrot was obtained and preserved in RNA*later*. The RNA extraction was done followed by qPCR for quantification of the FOXP1 and ZENK expression in the sample. Gel electrophoresis was done and result shows that there is presence of FOXP1 and ZENK in the parrot brain. Different RNA concentration was used which are 170 ng/ml, 17 ng/ml, 1.7 ng/ml, 0.17 ng/ml and 0.02 ng/ml respectively. The result was documented as cycle threshold (CT) value. In RNA concentration of 170 ng/ml, the lowest CT value was recorded which are 22.91 in ZENK and 23.39 in FoxP1 while RNA concentration of 0.02 ng/ml has CT value of 23.94 for ZENK and 23.99 for FOXP1. As the CT value is indirectly proportional to gene expression, we are able to conclude that genes are presence in high amount in a high concentration of RNA.

Keyword: songbirds, gene expression, ZENK, Foxp1, PCR

1.0 INTRODUCTION

Parrots are being define as the bird in the order of Psittaciformes, often vividly colour, short down curved hooked bill, grasping feet, raucous voice, found especially in the tropics and feeds on fruits as well as seeds (Oxford, 2014). They are characterized by distinctive vocalizations, lifelong capacity for learning and their adaptability to changing environmental conditions (Homberger, 2006).

Parrots are a popular choice of companion animals because of their ability to mimic many sounds such as human speech, song and laughter. Some of the popular choices of parrots as a pet include African Grey Parrot and Lovebirds which are very intelligent, affectionate and can be trained to talk and sing.

There are two phases of song learning process in birds. The first phase is the memorization phase which occurs when the birds listen and learn a song. The song learning process is influenced by the surrounding such as their owners and members of the same group. The song that was learned was stored in a long-term memory. After that, sensorimotor phase takes place. Sensorimotor occurs when the vocal output of the bird is matched with the memorized information. Further practice by the bird will makes the song becomes similar with the learned song. This is why the birds are able to mimic every sound and tone of the learned song (Mendoza, 2011).

In addition, songbirds and vocalization in a parrot are also controlled by few genes in the brain. Previous study has reported that FOXP1 are most likely related with speech disorder and vocalization. In a study done by Teramitsu et al, (2004) the expression pattern of FOXP1 and FOXP2 in human brain are similar to those in songbirds including their localization to subcortical structures that function in sensorimotor integration and control of skilled, coordinated movement. Other than that, the specific colonization of FOXP1 and FOXP2 found in several structures in the bird and human brain predicts that mutations in FOXP1 could also related to speech disorders (Teramitsu et al, 2004).

Indeed, when songbirds hear the song of another individual of the same species or when they sing, the MRNA levels of ZENK gene increased rapidly in the forebrain areas which involved in vocal communication (Mello and Ribeiro, 1998). Therefore, it is highly suggested that these two genes are most likely to be responsible for songbirds and vocalization in parrots.

1.1 OBJECTIVE

The objective of this study is to analyze and evaluate the brain gene expression associated with the vocalization of a parrot.

1.2 HYPOTHESIS

We hypothesized that the ZENK and FoxP1 gene expressions are significantly higher in the brain of a parrot.

2.0 LITERATURE REVIEW

2.1 Songbirds

Human is one of the species that depend on vocal learning to acquire their spoken language. Apart from human, songbirds and some other species of avian are also known to rely on vocal learning. Scientific study has been made on songbird since late 1950 by Thorpe and Marler. Their study has shown that birds that are taken from the wild either as eggs or nestlings and tutored with songs will produce the songs that resemble the tutor song. There are two phases of song learning process. The first phases is the memorization phase in which the bird will listen to a song and the song was stored in the long-term memory. The second phase is the sensorimotor phase in which the vocal output of the bird was matched with the stored song. As the bird is practicing on the same song, the song will gradually become similar with the learned song (Mendoza, 2011).

2.1 FoxP genes

Foxp2 is the first identified gene that is specifically involved in speech and language development in humans (Webb *et al.*, 2004). Foxp2 is a member of of the Forkhead (FOX) family of proteins. Foxp1 is the close homologue to Foxp2, and they share a similiar N-terminal domain that represses transcription of genes that are also affected by Foxp2. Therefore, there is high possibility that Foxp1 could interact with Foxp2 in other tissues and regions where they are expressed (Scharff *et al.*, 2004). The overlap in Foxp1 and Foxp2 expression observed in songbird suggests that combinatorial regulation by these molecules during neuronal development and within vocal control structures may occur. Other than that, Foxp1 and Foxp2 expression patterns including their localization in structures that function in sensorimotor integration are similar to those in human fetal brain (Teramitsu *et al.*, 2004) This highly suggests that these two genes are also responsible in song and vocal production in birds.

In a study reported by Teramitsu et al., (2004) they have recorded that Foxp1 gene is expressed within song circuit structures of zebra finches. They are clearly expressed in the song nucleus HVC and song nucleus RA. They also reported that Foxp1 expression in zebra finch striatum and thalamus partially overlaps with Foxp2. The Foxp1 and Foxp2 expression was examined in the developing brain of male zebra finches at day-1 (d1) after hatching and during the song-learning period at day-35 (d35). At d35, the characteristics expression patterns of Foxp1 and Foxp2 are presence with enhanced expression of Foxp1 in the song nucleus. The premotor song nucleus HVC exhibits strong Foxp1 expression while exhibits moderate expression of Foxp2. The striatal song nucleus, area X, exhibits enhanced expression of Foxp1 while Foxp2 are expressed at comparable or slightly higher than the surrounding area.

2.2 ZENK gene

ZENK are one of the possible mechanism related to the formation of song memories. They are rapidly induced in the brain of songbirds when they hear song playbacks. When songbirds hear the song of another individual of the same species or when they sing, the mRNA levels of the ZENK gene increase rapidly in forebrain area involved in vocal communication (Mello *et al.*, 1998). Other than that, ZENK mRNA induction has also been observed in several telencephalic song control nuclei as result of active singing behavior in captive as well as in wild songbirds (Jarvis *et al.*, 1997).

In study reported by Mello et al., immunocytochemical assay was used to determine the time course of ZENK protein expression and the brain distribution of ZENK protein after song playback was presented. Based on the study, ZENK protein is expressed in areas involved with song perception and production. In the end of the study, they reported that birds that sing upon hearing song show ZENK protein expression in several song control nuclei. Lastly, ZENK protein expression in song control nuclei only reflected by the birds that vocalized, but not in those that did not vocalize prior response to the song playbacks.

2.3 Real-Time Polymerase Chain Reaction (RT-PCR)

The quantitative measurement of specific mRNA species is of major importance for studies on gene expression (Bachmair *et al.*, 2002). It contains the fluorescent system which makes it capable of monitoring PCR product accumulation. It allows us to quantify differences in the expression level of a specific target between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels.

3.0 MATERIALS AND METHODS

3.1 Animals

A bird which is from the parrot family is being used in this experiment. A 3 weeks old, male, Lovebird (*Agapornis* sp.) which found dead was being presented to UVH for post-mortem procedure. A brain tissue was obtained and preserved in RNA*later* and then was stored under -20^{o}C.

3.2 RNA extraction

RNA extraction was done by using RNeasy Mini Kit Qiagen. This process is being done for purification of total RNA from animal cells, animal tissues, bacteria, yeast and for cleanup of RNA from crude RNA preps and enzymatic reaction. Firstly, up to 30 mg of the RNA*later* stabilized tissues are being added with 0.1 µL of β-mercaptoethanol (B-ME) mixed with 600 µL Buffer RLT. Then, the tissue was disrupted and homogenized by using TissueRuptor with the lysate in the Buffer RLT. Disruption and homogenization are 2 distinct steps in RNA extraction. In disruption procedure, there will be complete disruption of cell walls and plasma membranes of cells and organelles which are absolutely required to release all the RNA contained in the sample. Incomplete disruption will results in significantly reduced RNA yields. Meanwhile, homogenization is to reduce the viscosity of the lysates produced by disruption. Incomplete homogenization result in inefficient binding of RNA to the RNeasy spin column membrane which will reduce RNA yields.

Then, the lysate was being centrifuge for 3 min at full speed. Then, the supernatant was removed by pipetting and being transferred to a new microcentrifuge tube. Only this supernatant will be used in the subsequent steps. After that, 600 µL of 70% ethanol being added to the cleared lysate and mixed by pipetting followed by transferring up to 700 µL of the sample to an RNeasy spin column placed in a 2 ml collection tube. The spin column was centrifuge for 15 sec at 10000 rpm. The flow-through was discarded.

Next, 700 µL of Buffer RW1 was added and then centrifuged at 15 sec and 10000 rpm. This procedure is being done to wash the spin column membrane. The flow-through was discarded. Then, 500 µL Buffer RPE are added and centrifuged for 15 sec at 10000 rpm. Then, another same volume of Buffer RPE added and centrifuged for 2 min at 10000 rpm. In this step, the flow through was discarded together with the collection tube. A new 2 ml collection tube was placed on the RNeasy spin column and centrifuged at full speed for 1 min. The collection tube was discarded along with the flow-through.

The RNeasy spin column was placed in a new 1.5 ml collection tube and 40 µL ofRNase free-water being added directly to the spin column membrane and centrifuged for 1 min at 10000 rpm to elute the RNA. Then, the RNeasy spin column was discarded. The 1.5 ml collection tube which contain the RNA product are being kept in -80^{0}C to prevent degradation of RNA. RNA purification steps was done by using NanoQuant before it was used in the next steps which is RT-PCR.

3.2 Reverse-Transcriptase PCR

RT-PCR was done to amplify the RNA product. Primers set of the gene are being added together with other master mix required for RT-PCR. The primer sequence for FOXP1 gene is 5'-AAT GCT TTA CAG GCT-3' on forward action and 5' –GTT CAT CTT AAT CTC TG-3' on reverse action. The primer sequence for ZENK gene is 5'-ACC ACA TTC CCA TCC CCT TC-3' on forward action and 5'-GCC ACT TGG GTT TGA AAA GTG-3'. The volume of master mix used in this step is 25 µL. The cycling conditions for this PCR was shown in Table 1. This step was done to amplify the RNA with the target gene. RT-PCR was done using the SensoQuestThermalcycler.

Table 1: Cycling conditions for RT-PCR

FOXP1	STEPS	ZENK
45^{o}C, 45 minutes, 1 cycle	Reverse Transcriptase	45^{o}C, 45 minutes, 1 cycle
94^{o}C, 2 minutes, 1 cycle	Initial PCR activation	94^{o}C, 2 minutes, 1 cycle
94^{o}C, 30 seconds, 45-50 cycles	Denaturation	94^{o}C, 30 seconds, 45-50 cycles
49^{o}C, 1 minutes, 45-50 cycles	Primer Annealing	56^{o}C, 1 minutes, 45-50 cycles
68^{o}C, 2 minutes, 45-50 cycles	Polymerization	68^{o}C, 2 minutes, 45-50 cycles
68^{o}C, 7 minutes, 1 cycle	Final Extension	68^{o}C, 7 minutes, 1 cycle
4^{o}C, ∞	Cooling	4^{o}C, ∞

3.3 Gel electrophoresis

Gel electrophoresis was done to visualize the gene expression and therefore we can confirm the presence of gene. The gel was made by using 1.5 g of agarose powder mix with 100 ml of Buffer TAE. Then, it was being heated in the microwave at medium heat for about 5 to 10 minutes. 1 µL of gel stain was added and pour into gel tray. The well comb was placed then allowed it to cool down at room temperature. After the gel have harden, it was submerged beneath the TAE buffer. Then, the loading of the sample process takes place by adding 2 µL of loading dye mixed with 5 µL of the RT-PCR product. The electrophoresis was run at 85 volt for 45 minutes. Lastly, the gel was visualized under UV transilluminator.

3.4 Real-Time PCR (qPCR)

Real-Time PCR (qPCR) was done to quantify the amplification of RNA. Serial dilution of 1:10 was done to detect the quantification of RNA in five different concentrations. The initial concentration of RNA used are 170 ng/ml followed by 17 ng/ml, 1.7 ng/ml, 0.17 ng/ml, 0.02 ng/ml, and 0 ng/ml. The volume of mastermix used was 18 µL and was added with 2 µL of RNA. For 0 ng/ml RNA concentration, nuclease free water was used instead of RNA product Real-Time PCR (qPCR) process was done using the StepOnePlus™ Real-Time PCR Systems. The cycling conditions for this PCR was 48^{0}C for 30 minutes, 95^{0}C for 10 minutes, 95^{0}C for 15 seconds with 40 cycles, and 60^{0}C for for 1 minutes with 40 cycles. The result of qPCR was recorded as CT value.

3.5 Statistical Analysis

Analysis was done by using the qPCR results. The results obtained was recorded and analyze using the Microsoft Excel.

Figure 1 : Process of Reverse-Transcriptase PCR (RT-PCR) for ampilification of RNA using SensoquestThermalcycler.

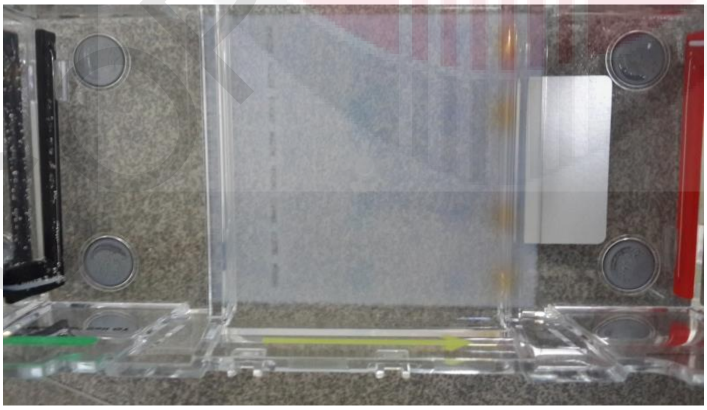

Figure 2 : Agarose gel electrophoresis procedure to detect the presence of ZENK and FOXP1 in the brain.

Figure 3 : Real-Time PCR (qPCR) process using the StepOnePlus™ Real-Time PCR Systems for quantification of RNA and gene expression.

4.0 RESULTS

From the gel electrophoresis results, it shows that both gene ZENK and FOXP1 are presence in the RNA. For the qPCR results, the highest concentration of RNA recorded the lowest CT value for both genes. For ZENK, at the RNA concentration of 170 ng/ml recorded CT values of 22.91. For FOXP1, at the RNA concentration of 170 ng/ml recorded CT values of 23.39. The results shows that these two genes are presence in high amount and concentration of RNA. The results are recorded in Table 1 and Table 2.

Figure 4 : Agarose gel electrophoresis shows the presence of both gene in the parrot brain.

Table 2 : Results of FOXP1 expression in Brain Tissue

RNA Concentration (ng/ml)	CT values
170	23.39
17	23.46
1.7	23.68
0.17	23.87
0.02	23.99
0	30.12

Table 3 : Results of ZENK expression in Brain Tissue

RNA Concentration (ng/ml)	CT values
170	22.91
17	22.92
1.7	23.00
0.17	23.43
0.02	23.94
0	30.54

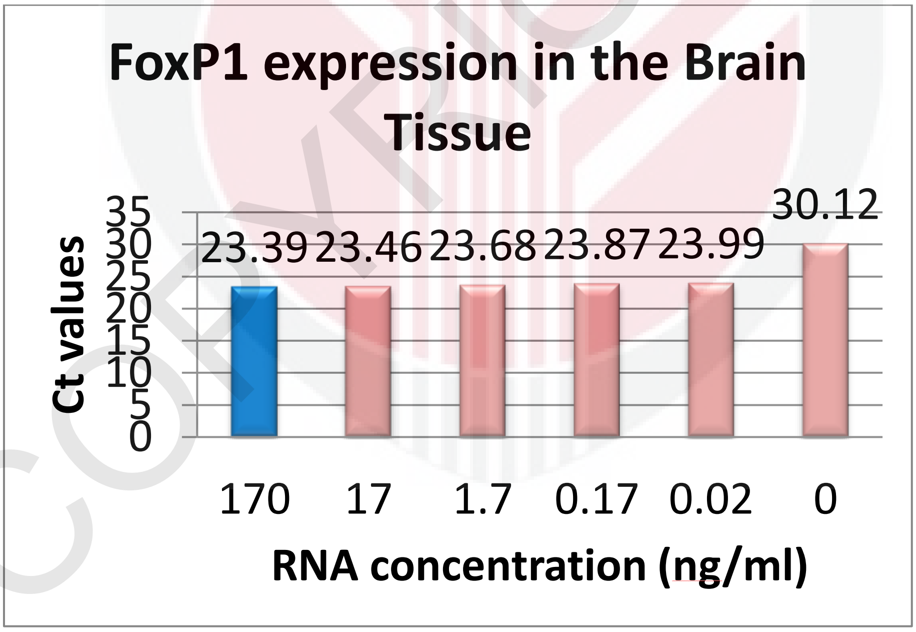

Figure 5 : Quantification of FoxP1 expression in parrot brain tissue using Real-Time PCR (Qpcr) with the starting concentration of 170 ng/ml followed by 1:10 serial dilution

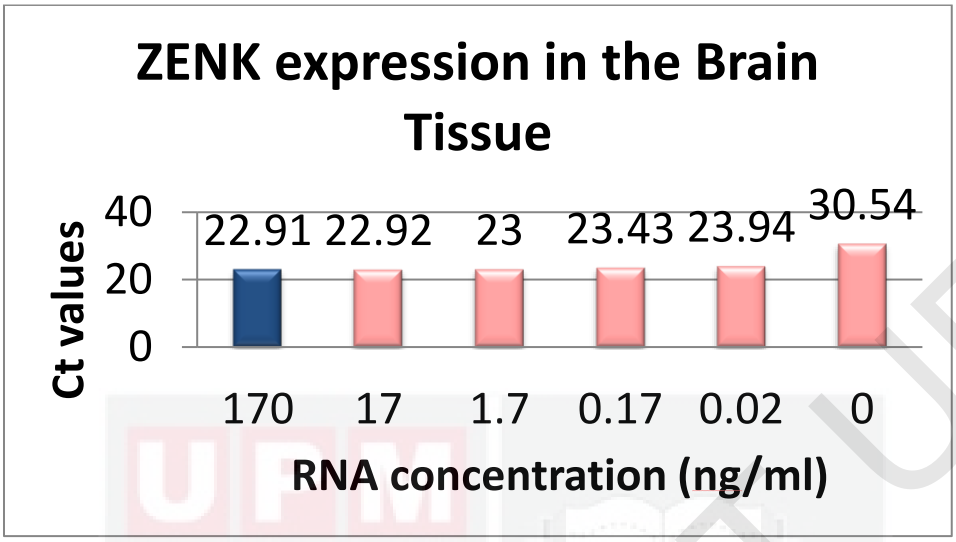

Figure 6 : Quantification of ZENK expression in parrot brain tissue using Real-Time PCR (Qpcr) with the starting concentration of 170 ng/ml followed by 1:10 serial dilution.

5.0 DISCUSSION

The present study showed that both genes ZENK and FoxP1 are presence higher in concentration of RNA. In the RT-PCR assay, a positive reaction is detected by the accumulation of fluorescent signal. The cycle threshold (Ct) value was used to shows the number of cycles required for the fluorescent signal to cross the threshold. The Ct values are inversely proportional to the amount of target RNA in the sample. Therefore, in this situation, the lowest Ct value indicates the highest amount of gene.

In this present study, the result shows that the highest concentration of RNA in both genes which is 170 ng/ml gives the lowest Ct value. The Ct value of less than 29 indicates strong positive reactions of abundant target nucleic acid in the sample.

Previous study has been reported that when songbirds hear a song or when they sing, the mRNA levels of the ZENK gene increase rapidly in the forebrain areas involved in vocal communication (Mello et al., 1998). In the study conducted by Mello *et al.,* (1998) immunocytochemistry was used to identify the levels and distribution of ZENK protein in the brain of zebra finches and canaries after presentation of song playbacks. In fact, birds that sing upon hearing the song have shown ZENK protein expression in several song control nuclei (Mello et al., 1998).

Meanwhile, FoxP has been identified as the gene underlying a human developmental language ability (Lai et al., 2001). In a brains of an avian and a primate vocal learner, both Foxp1 and Foxp2 have both distinct and shared expression patterns in the cortex, striatum, and thalamus (Teramitsu *et al.,* 2004). It was reported that the expression pattern of Foxp1 and Foxp2 in human fetal brain are similar to those in songbirds. Their localization to subcortical structures that function in sensorimotor integration suggests that it is highly related to speech disorder. They also reported that the expression of Foxp1 in two song nuclei are overlaps with Foxp2 and is higher than Foxp2. (Teramitsu *et al.,* 2014).

6.0 CONCLUSION

Parrots are one of the unique creatures as they are one of the songbirds which have unique ability in the cognitive function especially in song learning and vocalization. This study shows that ZENK gene and FOXP1 gene are responsible in controlling the vocalization and communications in songbirds as they are presence in high amount in the brain tissue.

7.0 RECOMMENDATIONS

As recommendation for further study, this study can be applied to detect the expression of ZENK and FOXP1 in different species of parrots or birds. Comparison can be done on the gene expression of FOXP1 and ZENK in different species of birds. Other than that, this study can be extended further by applying treatments on the parrots that may enhance the brain gene expression associated with the cognitive function such as omega-3 fatty acid. Dietary omega-3 fatty acid are proven to cause significant changes in expression of several genes in the central nervous tissue and can enhance brain cognitive function.

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