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Review

Sex identification methods of birds: a review

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Abstract: Birds are mainly categorized as either monomorphic or dimorphic based on phenotypic characteristics. It is believed that nearly 60 % of the bird species are monomorphic and difficult to distinguish the sex during their early stages of life. It is important to identify the different sexes at a distance when birds are in the wild or in natural habitats, especially for bird lovers, biologists and conservationists to differentiate the behavioral patterns of birds. However, even aviculturists and experienced biologists have faced difficulties in identifying the different sexes of monomorphic birds at a distance. The key genes responsible for the sex differentiation of chickens are unclear due to their multiple gene expressions. Therefore, the sex determination of birds is more important in aviculture, poultry farming and in research fields. Hence, this review mainly focused on those invasive (laparoscopy), moderately invasive/ minimally invasive (Karyotyping and DNA-based techniques), and non-invasive methods (steroid sexing, vent sexing, morphometric observations, voicing) of sex identification of birds as much as possible and discussed the reliability of those techniques on the identification of the sex of birds. In light of the discussion, the accuracy of non-invasive techniques is very low and quite questionable. Despite the high accuracy, laparoscopy poses a life threat to the birds during the surgery due to the damage of internal organs while probing. DNA-based methods are concluded to be the best and most accurate techniques among almost all the techniques that require sophisticated laboratory facilities. Hence, the on-farm approach of Recombines polymerase amplification combined with a lateral-flow dipstick (RPA-LFD) can be suggested as one of the best alternatives to laboratory protocols that can be practiced without bringing down the accuracy of the sex determination of birds.

Keywords: aviculture; bird; differentiation; dimorphic; monomorphic; poultry

1. Introduction

The scenario of determining the sex in animal kingdom exhibits an amazingly higher degree of variability though the accurate sex determination and differentiation are necessary for all sexually reproducing species for their survival (Wilhelm *et al.*, 2007). Birds can be differentiated into two main categories namely dimorphic and monomorphic are based on the general characteristics of male and female birds whereas dimorphic birds could be obviously differentiated into male and female based on their appearance (Lai *et al.*, 2022). There is no distinct sexual dimorphism in approximately 60 % of the bird species and it is hard to distinguish the gender solely by using the morphology (Griffiths, 2000; Wu *et al.*, 2007). As similarly in mammals, the sex determination of birds is strictly regulated by sex chromosome composition established at fertilization (Arnold *et al.*, 2013). However, the genes that involve in sex differentiation become active later in the development of birds (Lin *et al.*, 2010; Ayers *et al.*, 2013). Additionally, Berg *et al.* (1998) reported that the sex differentiation of vertebrates is extremely depends on the estrogen levels and the duration that takes for the differentiation process is influenced by the endocrine disruption chemicals. Monomorphic birds including many species of parrots, geese, and even cranes are difficult to differentiate based on their size as well as their body coloration which leads to many problems in captivity and /or in wildlife (Volodin *et al.*, 2015). Boersma and Davies (1987) stated that the sexing of monomorphic birds at a distance is seriously difficult even for biologists. However, in bird ecology, gender allocation, and sex ratio measurements are very important with a reliable and effective method of sex identification (Wu *et al.*, 2007). Correspondingly, sex differentiation is also very important in aviculture, scientific research as well the aspect of conservation of threatened species of birds. It would be beneficial while studying the bird populations, behavior, evolution, and wild fauna management. Furthermore, it plays an important role in improving the reproduction planning by analyzing the breeding strategies and additionally provides an added advantages in forensic cases (Lai *et al.*, 2022). Moreover, Chen *et al.* (2022) stated that the sex determination of chicken is very complex and precisely regulated by multiple genes. However, it is substantial to determine the sex of the poultry at day old stage since the female birds exclusively used for commercial layer production whereas the males used for broiler production due to its rapid growth patterns (Kaleta and Redmann, 2008). There are many methods used to identify the sex of birds including vent sexing (Bazzano *et al.*, 2012), laparoscopy (Richner, 1989; Cerit and Avanus, 2007), steroid sexing (Staley *et al.*, 2007; Blank *et al.*, 2020), chromosome inspection (karyotyping) (Cerit and Avanus, 2007), morphometry (Baehaqi *et al.*, 2018), acoustic method/ voicing (Volodin *et al.*, 2015), and novel techniques which based on DNA (Griffiths, 2000; Balkiz *et al.*, 2007; Cerit and Avanus, 2007; Wu *et al.*, 2007; Morinha *et al.*, 2012; Bosnjak *et al.*, 2013; Morinha *et al.*, 2013; Vucicevic *et al.*, 2013; Purwaningrum *et al.*, 2019). However, it's noteworthy to discuss the reliability as well as the accuracy of these methods in sex determination of birds. Concurrently, it's needful to discuss the contemporary topic of on-site approaches of sex determinations with high accuracy that will be a better alternative for expensive and time-consuming laboratory protocols of sex identification of birds.

2. Different sex identification techniques

2.1. Vent sexing

The slight differences in cloaca are a widely used technique in sex identification of birds among aviculturists all over the world. More dilated cloaca is observed during the laying season for ease of passage of eggs (Stromberg 1977). In this method, the presence or absences of the phallus (male reproductive organ) or presence or absence of the clitoris of female birds is experienced by pressing the cloaca and gently pulling to front with the thumb (Bazzano *et al.*, 2012). This method has been practiced in several species including penguins (Samour *et al.*, 1983), ratite birds (Samour *et al.*, 1984), greater Rhea chicks (Bazzano *et al.*, 2012) and Maleo birds (Widnyana *et al.*, 2019). It was found that this method was more successful in greater Rhea chicks with 98 % accuracy in determining sex without any welfare issues up to three months of age. It further suggested that this method is a good alternative in sex identification for greater Rhea birds when compared to molecular methods (Bazzano *et al.*, 2012). Vent measurements of American Coots and Magellanic Penguins also observed 100 % accuracy in differentiation of sexes within a few days after egg laying (Dee Boersma and Davies, 1987). Widnyana *et al.*, (2019) determine the sex of Maleo birds by using the presence or absence of protrusion on the top of the cloaca. The protrusion is visible in males whereas it is absence in female Maleo birds (Figure 1). Moreover, this vent sexing was experienced in sex differentiation of guinea fowls in Ghana. But the accuracy was very much less where 56 males and 81 females were correctly identified out of 115 males and 100 females, respectively with a calculated overall error rate of 36. 3 % (Ahiagbe *et al.*, 2018).

2.2. Feather sexing

Difference in feather characteristics which is determined by a specially selected genetic trait present in male and female day-old chick strains is the base of feather sexing (Figure 2). However, Otsuka *et al.* (2016) revealed a novel endoscopic technique which could be effectively used to differentiate day old chicks in poultry industry with an overall accuracy of 90.2 %. A probe is inserted through the cloaca until the intestine and check for the gonads is experienced under this method (Figure 3).

Figure 1. Protrusion on the top of the cloaca of male Maleo bird (A) and absence of protrusion on the top of the cloaca of female Maleo birds (B) (Source: Widnyana *et al.*, 2019).

Figure 2. Identification of male and female birds based on their rapid feathering wing feathers of females and slow feathering wing feathers of males (Source: Helton, 2022).

2.3. Morphometry

Eclectus parrots and the budgies are the easiest breeds among the pet birds in sex differentiation (Figure 4). Male Eclectus parrots are green color whereas the females are in red color while the cere of male budgies appeared in significant blue color, whereas it varies from pale blue to brown color in females. Besides, the naris of the female budgies encircled by a pale rim even though it isn't prominent in males and blue in color (Sakas, 2012).

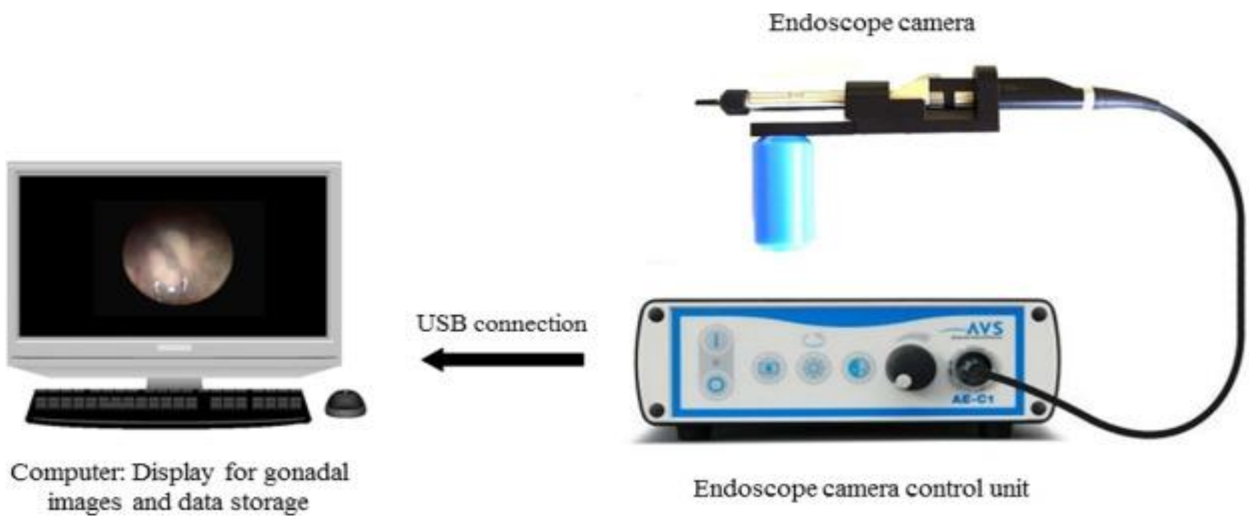

Figure 3. Determination of sex of day-old chicks by using an endoscopic technique (Source: Otsuka *et al.*, 2016).

Eye color is also one of a morphological characteristic of sex differentiation of some species of birds like cockatoos (Figure 5). It is found to be that the cockatoos with red iris are exactly female whereas the brown iris consisting ones might be either males or immature females (Sakas, 2012).

Figure 4. Male (Green color) and female (Red color) Eclectus parrots (Source: Martínez *et al.*, 2020).

The color of cere, chest and rump feathers as well as the mantle of parakeets (*Melopsittacus undulates*) were found to be the only accurate morphometric characteristics of sex differentiation whereas there were no any significant differences observed in length of body, upper bill, lower bill, wing, tail, femur, tibial-tarsus, tarsometatarsus as well as the body weight between males and females. The cere in blue in color of males and white color in females similarly in budgies (Baehaqi *et al.,* 2018). Lahaye *et al.* (2014) reveled that this was due to the effect of testosterone hormone. High concentrations lead to have more blue color in cere of male parakeets. Male chest and rump feathers are more blue in color than the females while the mantle primarily black in males and blue in females of parakeets (Baehaqi *et al.,* 2018). These finding were more or less concurred with the findings of Igic *et al.* (2016). Moreover, Hirschauer *et al.* (2018) reported that head morphometry can be used to differentiate male and female cape vultures (Figure 6). Head length, width and bill depth were used as the predictive variables and found that the males have wider and short heads as well as with larger bill depth than the females. Furthermore, Muriel *et al.* (2010) found that the discriminant function analysis could be used to differentiate the sex of birds. Though the forearm measurements aren't widely used in sex differentiation of birds, discriminant analysis of forearm and tarsus measurements were proved as better variables of sex differentiation in young Ospreys (*Pandion haliaetus)* by evidencing that the male Ospreys are smaller in size compared with the females. Moreover, Widnyana *et al.*, (2019) reported that the macrocephalon measurements which continuously grow until three years of age can use to differentiate males and females of Maleo birds where males have rounded macrocephalon with average 3.96 ± 011 cm width and females have oval shaped one with average 3.20 ± 0.10 cm width (Figure 7).

Figure 5. The difference between male and female Galah Cockatoo where the males have large curunculations around the eye with black color dark iris (left) and females have pink color iris (right) (Photographed by Rick Dawson) (Source: Saunders and Doley, 2019).

Figure 6. Sexing cape vultures using head morphometry (Source: Hirschauer *et al.*, 2018).

Figure 7. Sex determination of Maleo birds by using macrocephalon measurements (Source: Widnyana *et al.*, 2019).

Figure 8. Endoscopy-assisted minimally invasive technique of sex identification of birds (Source: Hernandez-Divers, 2005).

2.4. Pelvic sexing

Pelvic sexing also used in sex differentiation specially for pet birds even though its accuracy is quite questionable. The pelvic bones are palpated from the ventral side of the bird to notify the distance between those two bones where the distance is less in males compared to females (Sakas, 2012). Bugden (2022) reported that the mature Masked lovebirds over one year can be differentiated by using pelvic sexing. Moreover, it explained that the females show a significant distance between those two pelvic pumps to facilitate the egg laying.

2.5. Surgical sexing

An invasive techniques of sex identification of birds which associated with high risk and the effectiveness depends on the skills of the technician (Sakas, 2012). It is implemented with an optical telescope obviously with a tiny diameter and also suggested as a better alternative for laparotomy (Richner, 1989). Sex organs of birds are located near front portion of the kidneys (Sakas, 2012). A small lateral incision from the left side parallel to the posterior ribs of the bird is required for this procedure (Richner, 1989; Cerit and Avanus, 2007). Even though there is a risk associated with the anesthesia, the bird is anesthetized before starting the procedure for preventing pain (Sakas, 2012). Recently, miniature endoscopic equipment which are minimally invasive has been evolved and easily can be used in ornithology. Besides, other endoscope-assisted minimally invasive techniques (Figure 8) including enterotomy, enterectomy, cloacopexy and pneumotomy are also being evolved (Hernandez-Divers, 2005).

Swengal (1996) reported that non-vascularized and vascularized surfaced testicles in immature and mature males, respectively and granular surfaced ovary and follicle clusters in mature females are tried to observe in this surgical sexing technique to determine the sex. Despite of the harmful effects while probing, rapid and accurate diagnosis, reduced surgical stress and improved pulmonary function could be suggested as the advantages of this method (Hernandez-Divers, 2005). Richner (1989) also reported that there were no any significant effects on survival and body weight of birds after this technique.

2.6. Steroid sexing

Basically, the estrogen and testosterone (E/T) ratio in fecal samples of birds has been used to determine the sex of birds. The estimated values of the E/T ratio were 1.4 and 6.5 in males and females, respectively for 198 individuals from 12 orders including Anseriformes, Casuariiformes, Ciconiiformes, Colombiformes, Cuculiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Piciformes, Psittaciformes and Strigiformes. This work concluded that the E/T ratio was quite less in males compared to females. This method is a good alternative for invasive techniques with approximately over 70 % accuracy of sex determination even though there is a seasonal variation in considering hormones during breeding seasons of some orders (Stavy *et al.*, 1979). Staley *et al.* (2007) also confirmed that this fecal steroid hormone procedure is one of the best non-invasive techniques of sex differentiation of birds while assessing the gonadal and adrenal status of a bird especially in rare and threatened birds of prey. Furthermore, Blank *et al.* (2020) revealed that the mean fecal androgen levels during courtship were higher than those of during copulation and incubation in males of large bird of prey like Harpy eagles whereas the higher mean fecal estrogen concentrations were estimated during the courtship and copulation in females.

A comparison of the eggs of peafowls (*Pavo cristatus*) which were previously sexed for its embryos by using molecular techniques before hatching showed that there were significant differences in egg yolk steroids, including androgens between male and female eggs. Significant increasing levels of Androstenedione, testosterone concentrations in males than females and significant increasing of 5α- dihydrotestosterone and 17β-Estradiol concentrations in female eggs were determined, respectively (Petrie *et al.*, 2001).

2.7. Acoustic method/ voicing

This technique is predominantly based on the analysis of computer images of vocalization via spectrograms. Even though hearing natural voicing is also practiced, spectrogram or power spectra analysis of voicing is more reliable and unbiased in differentiating the sex of birds. Hence, this technique is applicable to sex identification of a vast range of bird species (Volodin *et al.*, 2015). There are two different types of voicing that have been already identified as songs and non-songs (calls), even though there is no uniform definition to distinguish those two from each other (Searcy and Yasukawa, 1996). The male songs were mainly identified as a part of sex selection and use to protect its breeding territory from other males (Catchpole and Slater, 2008; Searcy and Yasukawa, 1996). The basal frequency of voice is the discriminating acoustic variable between the calls of males and female birds which isn't affected by the vegetation or the distance in the wild (Matrosova *et al.*, 2010). For instance, male Little Spotted Kiwi (*Apteryx owenii*) produces a loud whistle call with a basal frequency of 2800 Hz which is 1.5 times higher than the female basal frequency (1800 Hz). Though some dimorphic characteristics were there in those kind of species, the technique is more beneficial when identifying those species with a far distance, especially in the wild (Digby *et al.*, 2013). But in case of white-faced whistling ducks (*Dendrocygna viduata*), this scenario is vice versa. The maximum fundamental frequencies of male and

female calls were recorded as 4500 Hz and 5300 Hz, respectively. Furthermore, there was a remarkable finding with the Cuban Whistling Ducks (*Dendrocygna arborea*) which stating that the male call consisted with a higher fundamental frequency with a secondary fundamental frequency in the spectra vocalization which resulted a biphonation as well (Volodin *et al.*, 2009).
Vocalization is also a good alternative for sex differentiation of day-old chicks in poultry farming compared to labor oriented manual sexing methods. A method has been already established by using vocalization end points by using three parameters: short-term energy, short-term zero crossing rate and duration. The determination of sex of day old chicks was succeed with the average accuracy of 91.25 %, 87.08 % and 88.33 %, respectively for each parameter (Cuan *et al.*, 2022). Tikhonov (1986) found that the male chicks of domestic hens' calls were always shorter and higher in basal frequency than that of female day-old chicks as well. However, the reliability of sexing the birds by using acoustic method/vocalization is depend on the species in which at least one acoustic variable isn't overlapped of values between males and females (Volodin *et al.*, 2015).

2.8. Karyotypes
Birds and reptiles are the closest relative to mammals though the birds recognized by their female heterogametic nature with one copy of Z chromosome and one copy of W chromosome whereas the males consist with two copies of Z chromosome (Tone *et al.*, 1982; Yasushi *et al.*, 1991). Yasushi *et al.* (1991) found that the 70 to 90 % of the W chromosome of chickens comprised of bend repetitive DNA sequences which led to form that heterochromatic nature in the nucleus. Furthermore, it was found that the W chromosome is smaller than the Z chromosome. This heterochromatic nature can be used to differentiate the sex of birds under microscopic observations. Culture sample of living cells from developing feather or blood cells treated with colchicine to stop cell cycling with more condensation can be used with the staining with C- banding technique to observe whether the presence or absence of heterochromatic nature by observing the bent repetitive DNA sequences which can only be observed in W chromosome (Stefos and Arrighi, 1971). Despite the high accuracy, there were certain disadvantages associated with this technique including time consumption for obtaining of cell cultures and not much accurately applicable to some of the species like Ostrich due to its low divergence of Z and W chromosomes (Jr *et al.*, 2002).

2.9. DNA based methods
Deoxyribonucleic acid DNA, is the molecule that contains the genetic material necessary for an organism to develop and operate its functions within the body. Polymerase Chain Reaction (PCR) is the base of most DNA techniques which begins with a single DNA molecule and produce 100 billion similar copies within a short period of time and found of this novel technique made the molecular sciences easier and more advance (Mullis, 1990).
DNA can be extracted by blood cells (Bertault *et al.*, 1999) or feather bulb (Balkiz *et al.*, 2007; Bello *et al.*, 2001). But it was suggested that the feather sampling is more worthy than blood sampling due to stress during sampling, complexity and tiny blood vessels in the early stages of life. A 0.5 to 1 cm portion from the tip of the feather is used for extraction due to DNA availability is confined into the feather bulb (Bello *et al.*, 2001). It was revealed that the molted feathers and feces also can be utilized for the extraction of DNA for sex identification (Ramón-Laca *et al.*, 2018). Moreover, it is noteworthy that the research proved the successive rate of DNA extraction was higher from swab sampling than that of feathers sample (Turcu *et al.*, 2023).
The female bird still has a distinct DNA nucleotide sequence that is specific to the W chromosome and can be utilized as a sex-linked identifier during sex differentiation of birds is the scenario behind all the DNA based techniques of sexing in birds (Griffiths, 2000). The size of the Z chromosome is greater because it is largely conserved, but the W chromosome is smaller since it has lost the majority of its genes over evolution (Djelic and Stanimirovic, 2004). With the timely development of technology from chromosome level to molecular level, the novel Chromo Helicase DNA (CHD) gene which is associated with Z and W chromosome was discovered by Griffiths and Tiwari (1995). It revealed that if the bird does not show the CHD-W gene in the genome, the bird should be a male. Because it was confirmed that the CHD-W gene is being the W-linked gene while CHD-NW is the non W-linked gene of birds. Balkiz *et al.* (2007) experienced that female flamingo chicks had only one band at CHD-W while CHD-Z product was evident in males. Vucicevic *et al.* (2013) also confirmed that the CHD gene is a universal marker which can be used to identify sex in birds. Moreover, Morinha *et al.* (2013) found that the CHD1Z and CHD1W genes consist very small difference in its size ranging from 2 to 44 bp. Even though there were some other genes used to determination of sex including Wpcki, EE0.6 genes, CHD is unquestionably and more significant among them in almost all the bird species with the exception of ratites (Griffiths *et al.*, 1996). Nevertheless, it is noteworthy that there was a novel finding of DMRT1 gene in ratites

specially in emu which can be used to differentiate sex in ratites. In the emu, a homologues of DMRT1 is associated with the Z chromosome, but not with the W (Shetty *et al.*, 2002). Huynen *et al.* (2002) also discovered and isolated a DNA sequence that has to be appeared in W chromosome and suggested as a sources of sex differentiation of ratites. Moreover, Z-linked *ZOV3* and the gene for the iron-responsive element-binding protein were used to test the homomorphic Z and W chromosomes of ostrich and emu by using Fluorescence In Situ Hybridization (FISH) analysis and found that those two genes can be markedly used to predict the sex of almost all the ratites (Ogawa *et al*., 1998).
As reviewed by Morinha *et al.* (2012), PCR based techniques including Single strand conformation polymorphism (SSCP), Restriction fragment length polymorphism (RFLP), Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Microsatellites, Allele-specific PCR (AS-PCR), Real-time quantitative PCR (qPCR) using TaqMan probes are also can be effectively used in sex differentiation birds. Wu *et al.* (2007) reveled a novel marker by using RAPD technique that can be used to determination of sex in three species of birds under family Columbidae. High resolution melting (HRM) analysis was also proved as one of best advanced post-PCR method which can be used to determine the sex of birds with high sensitivity and high resolution (Morinha *et al.*, 2013). All the above discussed techniques were performed under laboratory conditions with more equipment. Recombinase polymerase amplification combined with a lateral-flow dipstick (RPA-LFD) analysis was developed to determine the sex of juvenile pigeon as a less equipped on farm approach and obtained 100 % accuracy in sex determination. It can be practiced rapidly compared with the other PCR-based techniques without diminishing the accuracy of the determination as well (Lai *et al.*, 2022).

3. Conclusions

Even though the vent and feather sexing, pelvic sexing, steroid sexing, sexing by using morphometrical measurements and acoustic methods are non-invasive techniques of sex determination, reliability and accuracy of those techniques are still doubtable and need more experience to practice. Despite of the accuracy of sex determination by using laparoscopy, harmful effects are there even for the life of the particular bird which experiences the surgery due to the damage of internal organs while probing. Concurrently, there is a significant welfare violation associated with this technique. Karyotyping is one of the accurate methods with some limitations such as time consumption and applicability issues in species like Ostrich. Overall, DNA based method are concluded to be the best and most accurate techniques among almost all the techniques discussed during this review even though some of the cons are experienced such as requirement of more equipment with laboratory conditions, experience and the cost of performing. Recombinase polymerase amplification combined with a lateral-flow dipstick (RPA-LFD) can be suggested as one of best alternative to laboratory protocols which can be practiced with less equipment as well as the on-farm approach without bringing down the accuracy of the determination. Future studies will be suggested with RPA-LFD technique with on-site approach to determine the sex of variety of monomorphic birds at their juvenile stage to check its 100 % accuracy will be validating for all the tested varieties.

Data availability

Not applicable.

Conflict of interest

None to declare.

Authors' contribution

Siriwardana Tharinda Dhilshan De Silva: Conceptualization, Writing - Original Draft; Mylvaganam Pagthinathan: Conceptualization, Writing - Review & Editing; Indunil N. Pathirana: Conceptualization, Writing - Review & Editing; Suranga Bandara: Conceptualization. All authors have read and approved the final manuscript.

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