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RESEARCH ARTICLE

SURVEY OF SCID GENE AND IMMUNOLOGICAL STATUS IN ARABIAN MARES AND FOALS

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Abstract

Horses have been shown to have the deadly genetic condition calledsevere combined immunodeficiency (SCID). Animals with the condition are unable to produce specific antigens for immunological responses; as a result, they become extremely vulnerable to pathogenic pathogens and die before six months.The control and responses to pathogens of neonatal and young foals' immune responses differ from those of adults. The purpose of this study to determine carrier of SCID gene in Arabian foals and mares in addition assessing cellular and humoral immunity in Arabian foals and mares. Thirty-eight, Arabian foals and mares were examined.Hair samples were examined for detection of SCID gene by fragment analysis. While blood samples were collected for erythrogram and leukogram. Also, immunoglobulins including Ig E, Ig G, Ig A and Ig M. Also, SAA and fibrinogen were measured in serum and genes expression using Real Time-PCR were performed for IL-10, IL-6, TNFα and TLR 4 mRNA. All samples were homozygous and free from SCID gene. RBCS and monocytes showed significantly high value in foals compared to mares while, there were significant high value in MCV, IgG, IgM, IL-10, IL-6, TNF-α, TLR 4 mRNA expression in mares compared to foals. SAA and Fibrinogen showed non-significant difference in mares and foals.SCID diagnostic tests will aid in breeding programme selection to prevent carrier-to-carrier mating. Also assessing immunity in mares and foals is very important tools to determine the optimal immunological pathways and provide fresh preventative measures to ward off contagious diseases.

Introduction:-

A genetic condition that affects humans, horses, dogs, and mice is known as severe combined immunodeficiency (SCID) disease **(AbouEl Elaet al., 2018).**Arabian horses and their offspring are the main victims of equine SCID.It is a hereditary condition which a horse with this condition has an inadequate immune response since it is marked by a severe loss of immune cells like T- and B-lymphocytes **(Wagner, 2006).**

Equine adenovirus, Pneumocystis carinii, Cryptosporidium parvum, and Rhodococcusequi are among the secondary opportunistic infectious pathogens that affected foals become extremely vulnerable to them.Alarge majority of the

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affected foals with SCID typically pass away before the age of six months. Consequently, SCID is one of the most prevalent causes of Arabian foal mortality that is resistant to antibiotic therapy**(KUL et al., 2014).**

Since carriers do not exhibit any clinical symptoms, DNA testing is crucial for identifying heterozygous individuals. Accurate carrier identification is necessary to prevent carrier-to-carrier matings and the birth of clinically compromised foals**(AbouEl Elaet al., 2018).**

Adult horses and neonatal horses have different immune responses. The control of immunity and the reaction to antigens differ, although sharing similar components. IgM and IgG endogenous serum immunoglobulins are present in modest, non-protective quantities at birth in foals **(Tallmadge et al., 2009).**Due to the type of placenta in horse is placenta spuria, the transfer of immunoglobulins is restricted. Because of this, colostrum suckling during a foal's initial few hours of life is crucial. When compared to adults, newborn foals' immunological outcomes are different, and this is evidenced by reduced antibody and T-cell responses as well as altered cytokine levels**(Perkins and Wagner, 2015).**

It's crucial for newborn foals to have an innate immune system composed of already-existing or swiftly triggered defenses, whereas an antigen-specific reaction necessitates exposure to pathogens and postnatal development. The effectiveness of the immune response is governed by a complex network of direct and indirect processes, including interactions between various cells and cytokine-induced actions**(Migdał et al., 2020).**

Acute phase proteins such as serum amyloid a and fibrinogen concentrations are frequently employed in horse practice to diagnose and assess illness **(Pollock, 2017).**

The goals of the present study were to screen the Arabian horses for carrier prevalence of SCID. Also, compare between foals and mares' Arabian horses through assessing humoral response (immunoglobins A, M, G and E) and cellular response (WBCs, IL-10, IL-6, TNF- α and TLR-4) also through some acute phase proteins (Serum Amyloid A and Fibrinogen).

Materials And Methods: -

Ethical Approval/Animal Welfare:

The investigation was carried out in conformity with the biomedical research care standards and the Clinical Pathology Department of the College of Veterinary Medicine at Benha University's Ethics Committee in Animal Experiments accepted the experimental methodology (No. BUFVTM 21-10-22).

The usual vaccination and blood sampling of horses during this study, which was conducted with the farm's supervising veterinarian's permission, remained unchanged. In the equine stable, Immunization and sample gathering were carried out. Horses were kept in their stables throughout the trial, fed consistently throughout the off-racing season, andgranted access to an endless supply of water. The horses were thoroughly monitored for the appearance of any unfavorable illness symptoms after immunization and sample collection.

Experimental design:

38 healthy, Arabian horses participated in this study (19 foals from both sex and 19 mares). Foals range in age from two weeks to six months, while mares are between three and five years old. Foals weigh between 80 and 150 kg, whilst mares weigh between (200-300 kg).

Blood and hair sampling:

From the jugular vein, 38 blood samples were aspirated with a one inch, 18to20gauge needle (19 samples from foals and 19 samples from mares). For determination complete blood count (CBC) about 5 ml bloods was taken using vacuum tubes (Vacutainer®) which have 3.6 mg dipotassium EDTA **(Draeger, 2020)**. The same 5 ml used for separation of plasma by centrifugation of the samples using a centrifuge at 3000 revolutions per minute for 15 minutes and plasma were acquired using an automated pipette, put into Eppendorf tubes that were clean, dry, and labelled, and then preserved in the freezer until evaluation of fibrinogen **(Labelle et al., 2011).** Moreover, 5 ml of blood were drawn into a plain, clean, well-dried centrifuge tube to separate the serum for estimation of the biochemical parameters. After allowing the blood samples to coagulate, serum samples were extracted using a centrifuge at 3000 revolutions per minute for 15 minutes.Sera were acquired using an automated pipette, put into Eppendorf tubes that were clean, dry, and labelled, and then preserved in the freezer until evaluation of

immunoglobulins: Ig M , Ig G **(Buening et al., 1977)** , Ig E **(Hoffman, 1981)** , Ig A **(Staley et al., 2018)**andserum amyloid A **(Cywinska et al., 2013).**

According to hair samples**:** 38 Follicle hair samples (19 samples from foals and 19 samples from mares) used in this investigation.Hairs (7 cm long) were removed from the neck or tail skin's base. The samples were kept safe in a paper envelope with individual labels that was kept at room temperature. until use for detection of SCID **(AbouEl Elaet al., 2018).**

PCR for SCID gene:
DNA Extraction:
Using the Genomic extraction kit (G- spin TM Total kit, INtRON), genomic DNA was extracted from hair follicles in accordance with the manufacturer's instructions.

Polymerase Chain Reaction:
With a set of primers around the five base deletion site, polymerase chain reaction was carried out using the Applied Biosystems AmpliTaq Gold 360 Buffer Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA)**(Swinburne et al., 1999).**

Forward primer:
5′-AAG TTG GTC TTG TCA TTG AGC-3′

Reverse primer:
5′-TTT GTG ATG ATG TCA TCC CAG-3′

Thermal-cycling conditions of PCR:
Initial step (1 cycle) at 95˚C/ 10 min meanwhile, Amplification (30 cycles) consists of denaturation at 95˚C/ 45 sec, Annealing at 60˚C/ 60 sec, Extension at 72˚C/ 40 sec and final extension (1 cycle) at 72˚C/ 10 min.

Fragment Analysis:
according to **AbouEl Ela et al., (2018).**

1. After PCR is finished, prepare master mix for fragment analysis, which composed of 1.0ul of the undiluted PCR product, 10 ul Hi-Di Formamide and 0.5 ulGeneScan™ 500 LIZ Standard then Briefly spin the mixtures in a microcentrifuge.
2. After that heat at 95ºc for 2 min, and then at once place the tubes on ice for 2 min and place the samples into the Genetic Analyzer 3500 and the re-suspension was plated out onto an ABI 96-well optical reaction plate. Then the plate was covered with clean rubber septa and ensured that there were no bubbles at the bottom of wells.

Determining the size of the amplified Equine DNA:

3. The panel of SCID for Equine DNA is determined based on the 5 base pairs differences size using GeneMapper Software.

PCR for TNF-α, IL6, IL10 and TLR 4 genes:
RNA extraction:
The QIAamp RNA-blood extraction kit was used to extract RNA from whole blood (Qiagen, Germany, GmbH). The procedures were carried out in accordance with the QIAamp RNA-blood extraction kit's Purification of Total RNA protocol. Primers used were supplied from **Invitrogen by Thermo Fisher Scientific** are listed in table **(Vendrig et al., 2013).**

Target gene	Sequence
B- actin	F 5′-CAAGGCCAACCGCGAGAAGATGAC-3′
	R5′-GCCAGAGGCGTACAGGGACAGCA-3′
TNF-α	F 5′-TCCAGACGGTGCTTGTGC-3′
	R5′-GGCCAGAGGGTTGATTGACT-3′.
IL6	F 5′-TGGCTGAAGAACACAACAACT-3′
	R 5′-GAATGCCCATGAACTACAACA-3′.
IL10	F 5′-GAGAACCACGGCCCAGACATCAAG-3′
	R 5′-GACAGCGCCGCAGCCTCACT-3′.

TLR 4	F 5′-CCCTTTCAACTCTGCCTTCACT-3′
	R 5′-GGGACACCACGACAATAACTTTC-3′.

SYBR green rt-PCR
20 µl reaction consisting of 10 µl of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 0.5 µl of each primer of 20 pmol concentration, 1 µl of RT Enzyme Mix (20X), 0.5 µl dye, 2.5 µl of water, and 5 µl of RNA template. The reaction was carried out in a step-one real time PCR machine.

Conditions for SYBR green rt-PCR:
Reverse transcription was done at 45˚C/ 5 min. Also, Primary denaturation at 94˚C/ 30 sec meanwhile, Amplification (40 cycles) consists of denaturation at 94˚C/ 5 sec, Annealing at 55˚C/ 15 sec and Extension at 72˚C/ 10 sec.

Statistical analysis:
The statistical evaluation was completed using T-test through SPSS, ver. 25 IBM Corp. Released 2013 Data were handled as a complete randomization design according to **Steel and Torrie, (1980)**.The significance level was set at < 0.05.

Result: -

Blood and hair samples were collected from foals and mares and the results that were attained are as follows:

Fragment analysis for SCID gene:
Data showing fragment analysis for SCID gene are in fig (1-2)

In fragment analysis, labeled forward primer and unlabeled reverse primers covering the candidate mutation site are used. In control positive carrier, two different alleles were detected, first allele represented the mutant genotype at size 160 bp, whereas the second allele at size 166 bp represented the normal genotype. All studied samples were homozygous normal at the size of 166 bp.

Hematology of foals and mares:
Data showing hematology of foals and mares are listed in table (1)

RBCs and MCV in foals showed significantly very high value and significantly very low value respectively compared to mares. While Hb, HCT, MCH and MCHC showed non-significant difference.

According to WBCs, monocytes in foals showed significantly very high value compared to mares. While WBCs, Lymphocytes, eosinophils, and granulocytes. showed non-significant difference.

Immunoglobulins of foals and mares:
Data showing immunoglobulins of foals and mares are listed in table (2)

Ig G, Ig M, Ig A and Ig E in mares showed significantly very high value compared to foals.

Serum amyloid A and Fibrinogen of foals and mares:
Data showing SAA and Fibrinogen of foals and mares are listed in table (2)

SAA in foals showed significantly high value compared to mares while,fibrinogen showed non-significant difference in mares compared to foals.

Expression of TNF-α, IL-6, IL-10 and TLR-4 m RNA in foals and mares:
Data showing the expression of m RNA in foals and maresare listed in table (3)

Mares showed very high significant expression in TNF-α and IL-10 by 7.5 folds and 3.8 folds respectively, also showing high significant expression in IL-6 by 2.1 folds and showing significant expression in TLR-4 by 5.6 folds when compared to foals' group.

Discussion:-

Numerous genetic problems affect Arabian horses. One of the prevalent genetic disorders of Arabian horses is severe combined immunodeficiency (SCID), which is regarded as a deadly hereditary condition**(AbouEl Ela et al., 2018).**

By three months of age, the illness, which manifests as a T and B lymphocyte deficit in foals, is fatal due to infection with opportunistic infections **(Bailey et al., 1997).**Adenovirus-induced infections of the respiratory tract are the main cause of death. SCID-affected foals have proven challenging to maintain, although hyperimmune plasma infusions can extend their survival**(Lund, 2007).**

According to SCID gene,the pattern of peak created by the wildtype allele in fragment analysis was plainly visible at the predicted size of 166 bp, but the mutant allele measures barely 161 bp. This result agree with **AbouEl Elaet al., (2018)**who said thatthe wild-type and mutant alleles, with anticipated sizes of 166 bp and 161 bp, respectively, were heterozygous for two peaks in the pattern of peaks for the carrier control positive for SCID. In this study, the total evaluated samples displayed a single peak pattern of 166 bp in size.The SCID allele was found to be absent from our samples. Most breeders do not make genetic testing prior to breeding because most carriers of genetic disorders have no symptoms, are frequently inherited in a recessive manner, and are challenging to identify phenotypically**(Tryon et al., 2009).**

Adult horses and neonatal horses have different immune responses. Even though they both have similar parts, how immunity is controlled and how the body responds to antigens is different. Due to the modest concentration of immunoglobulins that foals are born with, infectious illnesses are frequent in them during the first and fifth months of life**(Migdał et al., 2020).**

In our research, foals are analyzed and compared to mares. This group of foals shows significantly very high value of RBCs and very low value of MCV ($p<0.001$) compared to mares. Our results concur with **Dekic et al., (2014)**who stated that foals had higher values of RBCs and lower values of MCV than mares.

In comparison to adults, newborn foals have larger RBC of foetal origin, greater RBC, and Hb levels.These parameters begin to diminish within the first 12 to 24 hours of life, then gradually decline over the following two weeks, reaching typical adult haematological values by 1 to 2 years of age(**Grondin and Dewitt, 2010)**. Both parameters initially increased due to the transfusion of placental blood to the foal at birth; the drop in RBC was caused by catecholamine release, and the fluid balances were adjusted due to the osmotic effect of colostral immunoglobulins' absorption.Increased blood oxygen levels, improved oxygen delivery to tissues, and haemoglobin saturation are all associated with reduced stimulation for erythropoietin production, which, in turn, reduces RBC survival time and iron delivery to bone marrow, resulting in lower RBCs in adults than foals **(Harvey et al., 1984).**

MCV are elevated during birth and then decline, reaching their minimum levels at 3-5 months of age. They never reach adult values. Microcytosis in foals has been connected to a drop in serum iron due to an increased need for growth**(Faramarzi and Rich, 2019)**.Some impedance counts may not be able to identify these RBCs because of their small size. In young foals, mild anisocytosis is also a common observation. After delivery, MCHC remains steady and resembles adult values **(Satué et al., 2012).**Also, Hb and Hct are high at birth, fall quickly within the first 12 to 24 hours of life, then diminish more gradually over the following two weeks, staying within the lower range for adults after that **(Grondin and Dewitt, 2010).**

Leukogram results indicate, there is no significant change in whole WBCs except monocytes.Our results concur with **Lawrie, (2012) , Satué et al., (2012) and Barton and Hart, (2020)**who stated low levels of neutrophils are present in the fetus, but they rise in response to cortisol after delivery and then at about 4-6 months of age, decline to mean adult values, healthy foals have a band NEU that doesn't go over 150/l.In foals, LYM levels start out low at birth, increase at three months, and then increase to adult levels by one year**(Satué et al., 2012).**EOSs are not typically found in fetus and newborn foals, with a mean of 400/l by 4 months of age. Due to lifelong exposure to allergens, the EOSs count rises with age**(Satue et al., 2009).** Moreover**, McFarlane et al., (1998)**and **Hernández et al., (2008)** could not detect any variations in the number of EOS between foals and adult horses. AlsoHorses' basophils do not appear to alter with age**(Čebulj- Kadunc et al., 2003).**

According to monocytes, foals show significantly very high value ($p<0.001$) of monocytes compared to mares. This result concur with **Čebulj- Kadunc et al., (2003)**who stated that mares monocytes in foals showed significant higher value than mares group.

According to SAA,foals showed significantly high value ($p<0.05$) of SAA compared to mares. This result contrary to **Pollock et al., (2005)**who stated that foals have been shown to have similar baseline of SAA compared with adults.

Concerning to fibrinogen, thereare no significant changes between foals and mares' groups. Our result concur with **Fonteque et al., (2015)** who stated that foals have been shown to have similar baseline of fibrinogen compared with adults and plasma fibrinogen were not influenced by age .

In a general sense, In the majority of cases, SAA can be used to detect inflammation early on, evaluate the efficacy of a chosen antibiotic or other treatment, track the progress of a patient's recovery, and indicate when a condition has resolved**(Nolen-Walston, 2015).**

Every foal is born lacking humoral antibodies **(Hardy and Rakestraw, 2002).**Within a few hours of birth, they are dependent on the adequate consumption of high-quality colostrumto supply more IgG and lesser amounts of IgM and other immunoglobulin typesto offer effective short-term defence against infectious pathogens **(Eggleston and Mueller, 2003).**

According to immunoglobins, mares showed significantly very high value ($p <0.001$) of mean concentration of IgG, IgM, IgA and IgE compared to foals. This result agree with **Perkins and Wagner, (2015), Sedlinská et al., (2006)**and **Perkins and Wagner, (2015)** who stated that adults are higher than foals in immunoglobins.

Cytokines are potent modulators of innate and adaptive immune responses. The proper development of immunity and disease prevention depends on their fine-tuning. However, cytokines interact with cells in a variety of ways, leading to a complex web of activating and suppressing mechanisms**(Acosta-Rodriguez et al., 2007).**

According to TNF-α and IL 10 mRNA, the expression of TNF-α and IL10 mRNA in mares are significantly very high values ($p <0.001$) compared to foals. This result agree with **Mérant et al., (2009)**who stated that TNF-a and IL-10 expression was lower in foals than mare.

Following immunisation and early in life, throughout the first year, cytokine expression in young differs from that of adults and plays important functions in controlling adaptive immune response to infectious illness.For three months to a year following birth, in particular, T cell and cytokine responses in foals are slowed and delayed. According to IL 6 **,** the expression of IL6 mRNA in mares are significantly high ($P< 0.01$) compared to foals .This result agree with **Liu et al., (2009)**who stated that newborn foals' greater vulnerability to bacterial infections is caused by their decreased IFN- and IL-6 production.Poor age-dependent stimulus-induced Th-1-based and pro-inflammatory cytokine production in these foals can be used to explain why they have a reduced ability to respond to microbes, as seen by the decreased IFN-, IL-6, and IL-12 production in foals.

Toll-like receptors can detect PAMPs generated by bacteria, viruses, fungi, and protozoa. TLRs are expressed by different immune system sentinel cells, other leukocytes, and specific epithelial regions**(Akira et al., 2006).**

According to TLR 4, the expression of TLR4 mRNA in mares are significantly high ($P < 0.05$) compared to foals .This result concur with **VAN MECHELEN, (2014)** who stated that some foals are more susceptible to R. equi infection because of low serum opsonic activity, insufficient TLR signaling, and dendritic cell expression of CD86. Additionally, it is known that defects in innate cell activity might result from TLR signaling dysfunction in neonates.Poor generation of Th1-type cytokines including TNF- and IL-12 is the primary cause of poor newborn TLR-4 responses **(Belderbos et al., 2009).**

Conclusion:-

Our long-term objective is to prevent carrier-to-carrier matings and the delivery of SCID foals that are clinically afflicted. Based on the results of evaluating a selection of highly questionable samples and the findings given in this study, the possibility of the mutant allele being prevalent in the Egyptian population is very low.

Foals' immune system are not fully formed, as evidenced by the low concentration of TNF-, IL-6, IL-10, and TLR 4 mRNA in cellular immunity. Additionally, humoral immunity, which has a lower immunoglobin concentration than in mares, makes newborns more susceptible to infection.

Table (1):- Erythrogram and leukogram of mares and foals:

Parameters	Mares	Foals
RBCs ($x10^{6}$/µl)	**8.04±0.15**	**9.58±0.41*****
Hb (g/dl)	**11.01±0.20**	**11.08±0.26**
HCT (%)	**33.64±0.62**	**32.12±1.29**
MCV (fl)	**42.69±0.48***	**35.04±0.67*****
MCH (pg)	**13.86±0.17**	**13.18±0.95**
MCHC (%)	**32.39±0.16**	**32.29±0.10**
WBCs ($x10^{3}$/µl)	**8.02±0.29**	**8.99±0.53**
LYM ($x10^{3}$/µl)	**4.45±0.19**	**4.69±0.38**
MON ($x10^{3}$/µl)	**0.24±0.03**	**0.47±0.05*****
EO ($x10^{3}$/µl)	**0.32±0.02**	**0.42±0.07**
GR ($x10^{3}$/µl)	**3.27±0.24**	**3.99±0.32**

: Non- significant *: Significant ** high significant ***: Very high significant

Table (2):- Immunoglobins, SAA and Fibrinogen of mares and foals:

Parameters	Mares	Foals
IgG (g/l)	**522.34±11.31**	**329.36±36.66*****
IgM (g/l)	**123.82±8.84**	**18.42±2.15*** **
IgA (g/l)	**9.92±0.40**	**6.51±0.56*****
IgE(g/l)	**0.27±0.09**	**0.10±0.07*****
SAA (mg/l)	**2.682±0.69**	**4.294±1.612***
Fibrinogen (mg/l)	**21.96±2.127**	**25.46±3.504**

: Non- significant *: Significant ** high significant ***: Very high significant

Table (3):- Genes expression of mares and foalsby fold change:

Parameters	Mares	Foals
TNF-α	**7.50±0.49**	**1.00±0.00*** **
IL-6	**2.16±0.24**	**1.00±0.00** **
IL-10	**3.88±0.21**	**1.00±0.00*** **
TLR-4	**5.675±1.113**	**1.00±0.00***

: Non- significant *: Significant ** high significant ***: Very high significant

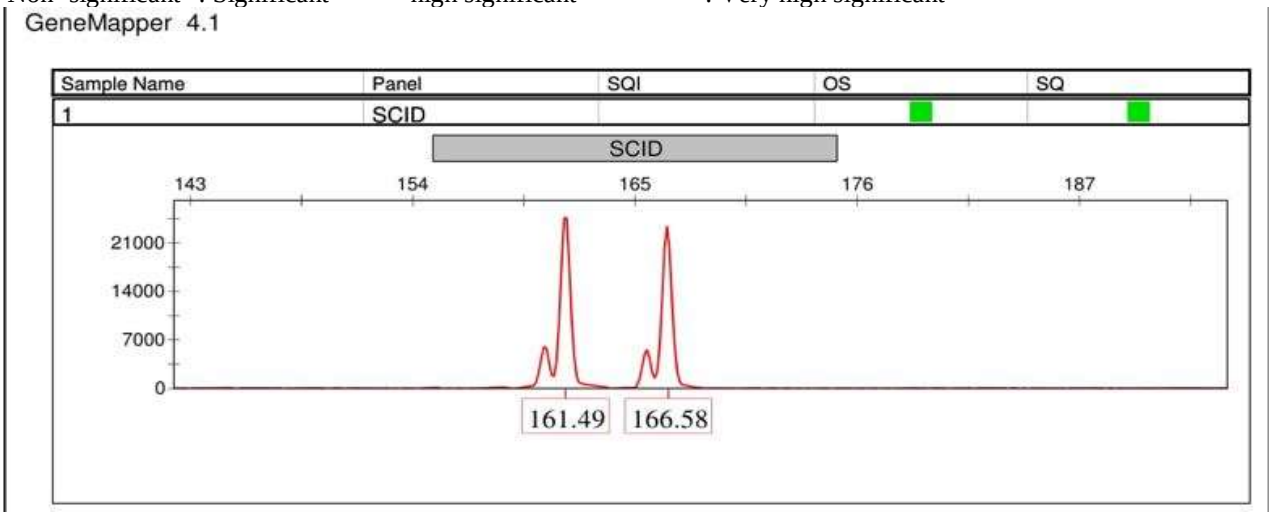
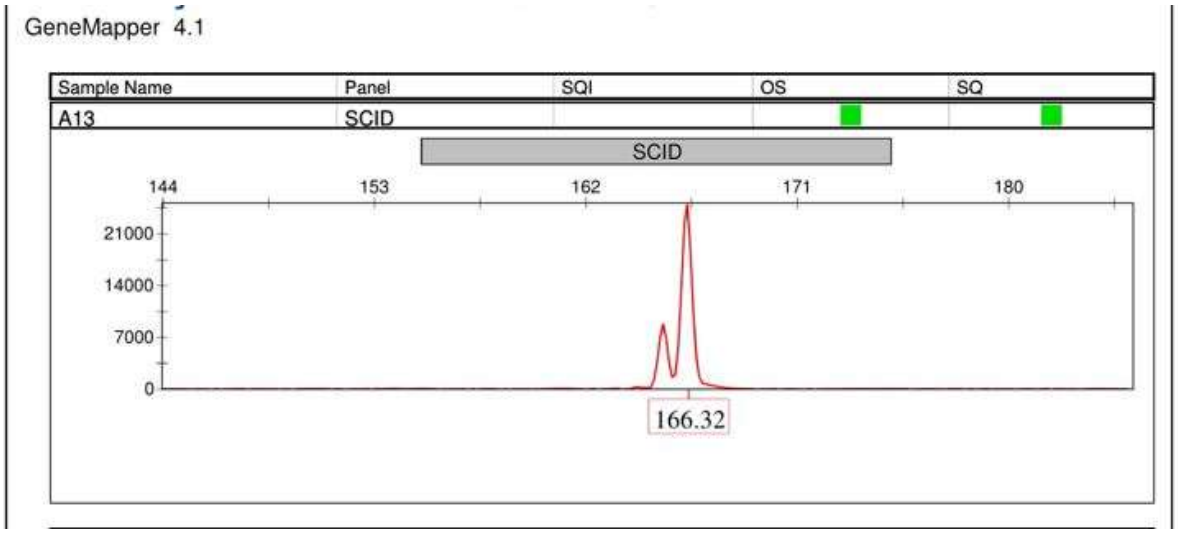


Fig (1):- Control carrier of SCID, shows two different peaks at sizes 161bp and 166bp representing both mutant and wild alleles respectively.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OS
SQ
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SCID
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153
162
171
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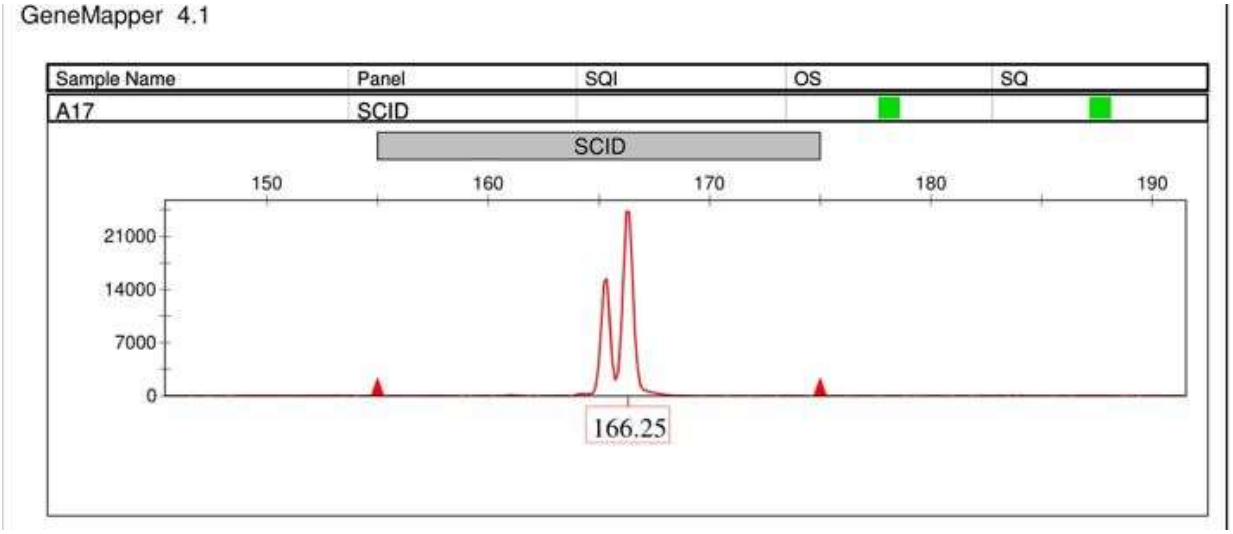
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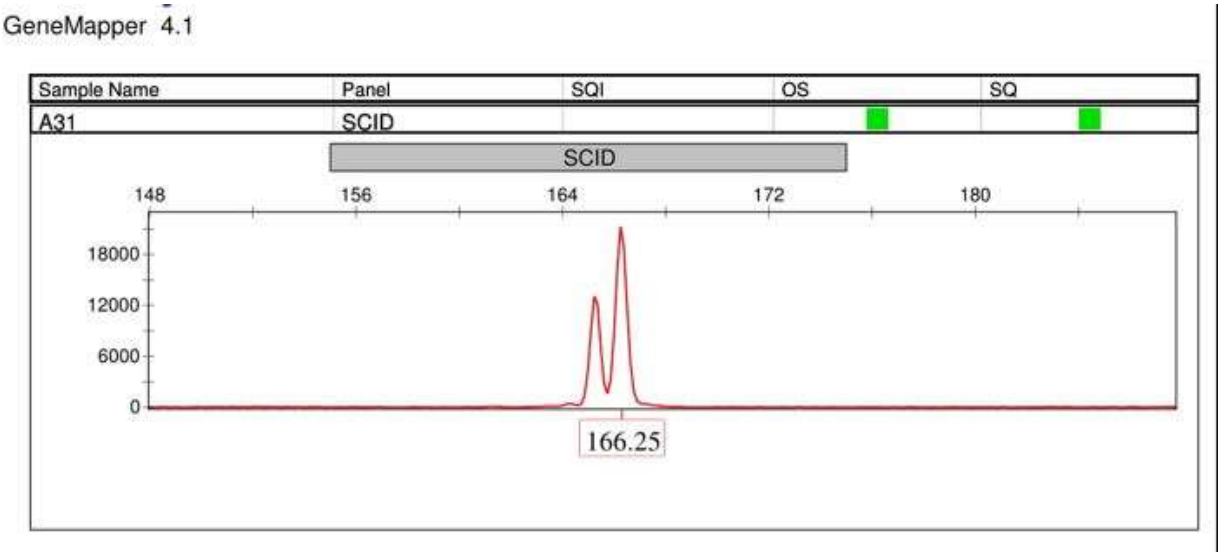
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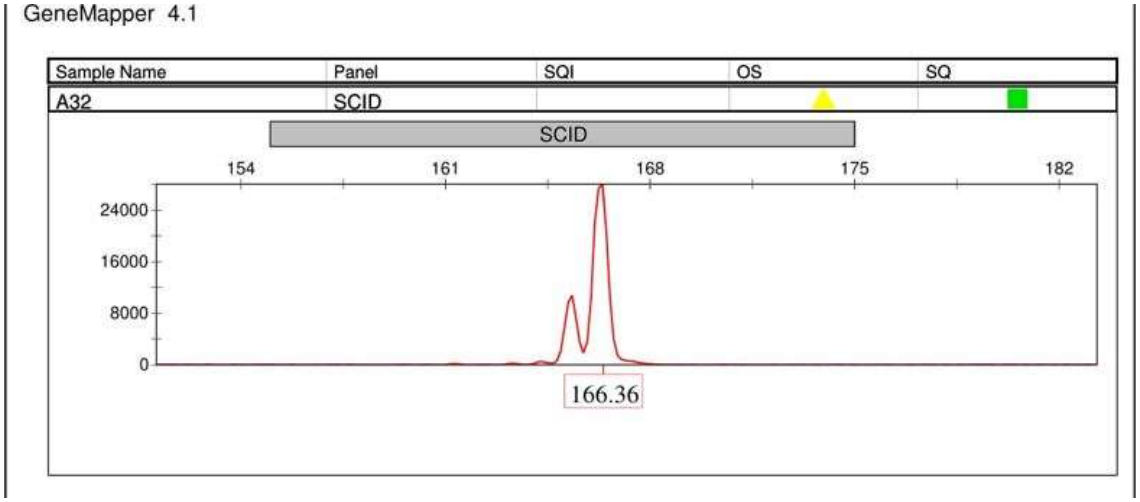

Fig (2):- Homozygous healthy samples, shows single peak representing the wild allele at 166bp.

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