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Contents

Front Cover: **ROSE-RINGED PARAKEET** ***(Psittacula krameri)***

The **Rose-Ringed Parakeet** (*Psittacula krameri*), is a medium-sized parrot in the genus Psittacula, of the family Psittacidae. The parrots, brought to Europe and Israel as pets, have established numerous wild populations. Since the 19th century, the rose-ringed parakeet has successfully colonised in many countries. In the wild, rose-ringed parakeets usually feed on buds, fruits, vegetables, nuts, berries, and seeds. Both males and females have the ability to mimic human speech.

Mr. Moshe Tachnai generously provided the pictures for the covers.

Editorial

Dear Readers,

This June edition of the Israel Journal of Veterinary Medicine (IJVM) holds many interesting and noteworthy articles.

The first written by Dr. Pozzi and Prof. Gardella Tedeschi delve into to the religious- ethical aspects of using animals within the domain of Judaism. Information spanning from the first chapters of the Bible and going through all the Five Books of Moses, and on to introducing the Jewish Oral Law as interpreted by learned Rabbis throughout the generations, is covered in this article. The article deals with all animals including working farm animals and to the slaughter of animals for consumption. Dr. Pozzi has written extensively on this subject and published a number of articles in the Israel Journal of Veterinary Medicine. I welcome Prof. Bianca Gardella Tedeschi and thank her for her contribution to the article on the "Use of animals in Jewish Tradition". Prof. B. Gardella Tedeschi is Associate Professor in Comparative Private Law, University of Eastern Piedmont. She concluded her Master of Laws at Harvard Law School, Cambridge, Mass. (U.S.A.), and then a Master degree in Cultural Anthropology at the University of Turin, Italy. She has dealt with private law, with a focus on gender equality, legal status of elderly people. A third field of research includes, within comparative religious rights, insights into Jewish law.

I regard this article as a valuable contribution from expert contributors and I suggest that our readers study this article.

A milestone article by Dr. Lublin describes the history of highly-pathogenic avian influenza in Israel. As he points out and emphasizes in the article "Avian Influenza has become the largest animal epidemic in the world". This alone should makes this article of interest to all our readers especially taking into account the public health elements of this disease.

The team led by Dr. B. Perelman never lets us down with his interesting and relevant ideas. Definitely a veterinarian thinking "out of the box" and bringing novel solutions to many aspects of the poultry industry.

Dr. Abayli *et al.* describes the re-emergence of Bovine Ephemeral Fever in Turkey. This article investigates the genetic properties of the virus and highlights the close association that countries in the Middle-East have to the spread of important animal diseases such as this one. Furthermore, I wish to point out that the scope of the IJVM has been defined as covering the Mediterranean Basin, a scope which is well warranted and justified as demonstrated in this article.

Finally, of great interest is the first isolation of Canine Kobuvirus in India by Dr. Agnihotri, *et al.* The virus has not been demonstrated here yet but should be of interest to veterinarians in Israel.

All the very best to our readers. Your feedback on articles or any general comments regarding in the journal is welcome.

Sincerely,

Dr. Trevor (Tuvia) Waner

Editor-in-Chief, Israel Journal of Veterinary Medicine

Use of Animals in Jewish Tradition

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ABSTRACT

On June, 1964 a Commission was appointed in Scotland with the purpose of "examining the conditions in which livestock are kept under systems of intensive husbandry and to advise whether standards ought to be set in the interests of their welfare, and if so what they should be". The Commission concluded its work recommending that for farm animals, at least 5 basic conditions, or freedoms, should be granted: Freedom from hunger and thirst; from discomfort; from pain, injury, or disease; from fear and distress; and to express normal behavior. In Judaism these concepts were already known for the last 32 centuries, being included in the Law (The Torah), even if otherwise declined; extensively commented by Sages thru generations until today; still considered valid and ethic; even influencing the Legislator or the Judiciary power. Protecting animals from harmful or dangerous situations, and minimizing any traumatic event, abstaining from any intentional traumatic action, and abstaining from any unnecessarily painful action, these are the basic teaching of Judaism relative to animal welfare. Judaism sees in animal's protection a powerful teaching for the respect of all the Creation and its protection. Granting the animals the satisfaction of their needs does not mean placing them at same level of man. In fact, their use is allowed, while highly regulated; their killing is also permitted in order to supply food to man; and again their killing must be done in a painless manner possible. Judaism developed a highly skilled and regulated way, the shechita, for slaughtering animals considered for food production, aimed to be quick, precise and as painless as possible; exclusively using skilled professionals. Today, in several European Countries, shechita is under scrutiny, with erroneous accusations of malpractice. In other Countries, like the USA, it is legally protected and clearly included among the humane ways of slaughter which are granted to be used.

Key Words: Animal Welfare; Protection; Needs; Freedoms; Suffering; Legislation.

INTRODUCTION

The Agenda of the Organization of United Nations (ONU) for Sustainable Development has been subscribed on September 2015 by 193 member States of The United Nations. The Agenda represents the action plan relative to human wellbeing and prosperity and world health (1). The program for the achievement of Sustainable Development goals started in 2016, and consists of a work program with 169 goals, to be achieved in the next 15 years. Countries are in fact, committed to reach these goals by 2030. Within the 17 goals for a Sustainable Development, goals 2, 12 and 15 ponder:

Goal 2: End hunger, achieve food security and improved nutrition and promote sustainable agriculture;

Goal 12: Ensure sustainable consumption and production patterns;

Goal 15: Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss.

The final objectives of these goals have a strong ethic and moral significance, with evident repercussions relative to the management of the planet's resources. Whereas, at least for

Goal 2, food safety is represented by achieving enough animal and vegetal productions destined for food by their distribution worldwide; with implicit considerations relative to "how" and "how much" we can use and exploit these resources.

Beyond technical aspects relative for how to carry out productions (vegetable, animal, energetic, etc.), it should be taken into account how technical choices or decisions are inevitably linked to cultural, and perhaps even moral, attitudes or perceptions possibly developed by a culture or faith towards that kind of act/production.

For example, not so far back in history, and in some western countries, which we consider culturally "close" to us, crop production could have an intrinsic value definitely higher than the intrinsic value of a slave involved in production of the crop, or, vice versa, the value of a slave was only in relation to his tasks. The issue we would like to present is whether there is a Jewish point of view relative to animals, and food production and safety linked to use of animals. Is Judaism referring to the animal world, to the environment or to food safety?

The issue itself of a relationship between ethic(s) and animals is contraversial! "Traditional theories of Christian, Kantian, Cartesian, or Aristotelic origins, argued that only humans are entitled to a higher moral status, whereas animals are not; this because humans own rationality, language and ability to act morally, differently from animals" (2). Until the 17th century, modern philosophers still believed animals as unconscious "*automata*" (a doctrine for which animals are mere machines) and even unable to suffer pain (3). In such a way, these theories consider part of the Creation (the animal world) merely from an utilitarian point of view. To be noticed, however, that was the Jewish philosopher Spinoza, in his "Ethica", on 1677, contested this view, recognizing in animals as sentients and possessing some more rights, even if he does not claim equal rights with humans (3).

ANIMALS AND THE ENVIRONMENT IN JUDAISM

Animals have been created day 5th and 6th of Creation, before man: "he was created on the eve of the Sabbath, so that if a person becomes haughty, God can say to him: The mosquito preceded you in the acts of Creation, as you were created last" (4).

To human kind it was said: "Fill the earth and master it and rule the fish of the sea, the birds of the sky, and all the living things that creep on earth" (5). In addition: "God settled mankind in the garden of Eden, to till it and tend it" (6); and He said: "See My creations, how beautiful and exemplary they are. Everything I created, I created for you. Make certain that you do not ruin and destroy My world, as if you destroy it, there will be no one to mend it after you" (7).

Since the beginning, so far, man was placed ahead of animals and creation: firstly as a ruler and secondly as a keeper of the latter.

The above does not preclude in anyway, the usage both of animals and environment; Abel was a shepherd, Cain a (agricultural) farmer.

Man was entitled:

- to rule over animals ("…*and rule*…") and to use them: for milk, work, leather; and where killing is practiced, under certain conditions: In fact Abel made sacrifices using animals.
- to use the land; in fact, Cain farmed vegetables and fruits.

But, at this stage, man was not yet allowed to eat meat (8, 9, 10, 11, 12, 13), a situation which lasted until the Great Flood. Talmud quotes: "Rav Yehuda quotes that Rav (Abba Arecha) says: Meat was not permitted to Adam, the first man, for consumption" (14).

Was this the ideal situation?

Cain thought that the level of the man was as the same as that of animals, therefore he brought vegetables as sacrifice (15). God did not ask, at that time, for sacrifices (16); Cain resolved this. Abel imitated him; "As a result of each having his own vocation, they each brought different offerings" (17). This situation induced Cain to be jealous, upset and downcast (18), where jealousy lead to murder.

Following the Great Flood or Deluge, the situation totally changed, and to Noah's descendants the explicit permission to eat animals was given: "Every creature that lives shall be yours to eat; as with the green grasses, I give you all these" (19). The reason why God permitted eating creatures (after they had been killed) was that all of them had to thank man for having kept them from perishing during the deluge. As a result, all the animals were now totally at the mercy of man" (20). Interestingly indeed, the right of eating animals comes after, and derives from, having firstly protected, and saved them (against the Great Flood).

STATUS OF ANIMALS AFTER THE REVELATION OF THE TORAH

In the Torah several commandments exist relative to animals.

One of them is particularly emphasized by Sages: "When you see the ass (donkey) of your enemy lying under its burden and would refrain from raising it, you must nevertheless help raise it" (21).

"Rava says: "From the statements of both of these *tannaim* (Sages whose views are recorded in the Mishnah) it can be learned that the requirement to prevent suffering to animals is by Torah law" (22). Rashi comments: "Can you possibly see his ass (donkey) crouching beneath his burden and forbear to help him? Thou shalt surely help him to unload the burden" (23). "You are warned not to remain inactive when faced with the animal's distress, even if its owner is your enemy" (24).

According to Judaism, from here come both the obligations to help or save an animal (positive/active commandment), and abstain from performing harmful or painful actions on animals (negative/abstain commandment). Saving an animal from a harmful or dangerous situation, minimizing any traumatic event, are considered obligations from the Torah (*mitzwa me-de-Oraita)* (25), such as exemplified by the overloaded/collapsed donkey. Abstaining from any intentionally traumatic action, abstaining from any unnecessarily painful action, are considered obligations instituted by the Sages (*mitzwa me-de-Rabbanan*) (26, 27).

Even if its importance has declined over time with respect to the past, the use of animals remains permitted, but highly regulated, with precise obligations to ban exploitation and abuse, which clearly have (also) an educational purpose. As example, we can cite: "You shall not plow with an ox and an ass (donkey) together" (28): "God had mercy on his creatures. An ass (donkey) is not as strong as an ox" (29). "You shall not muzzle an ox while it is threshing" (30): "It is from the roots of the commandment to teach ourselves that our souls should be a virtuous choosing what is right and cling to it, following kindness and mercy" (31).

Obligation to Feed the Animals: "I will also provide grass in the fields for your cattle and thus you shall eat your fill" (32). "One is prohibited from eating before feeding his animals, as it is stated: "And I will give grass in your fields for your animals first, and only then, you shall eat and be satisfied" (33). Note that referring to animals precedes that of the man.

Obligation to Rest the Animals: "Six days you shall do your work, but on the seventh day you shall cease from labor, in order that your ox and your ass may rest, and that your home-born slave and the stranger may be refreshed" (34). A gloss/commentary on this verse adds a further significance, beyond the mere "resting from work", and it quotes: "(*this verse*) means: give the animal some satisfaction, by permitting it to pull up and eat grass from the ground as it pleases. Or, perhaps, this is not the meaning but it indicates that it must rest. That one must tie it up in its stall so that it does no work in the field! You will, however, admit this is no satisfaction but a source of cruelty" (35). The gloss/commentary recognizes that even work animals can enjoy from Shabbath satisfaction/*oneg shabbath,* beyond the simple abstention from work.

Obligation to Give Refuge and Return (to their Owners) Lost Animals: "When you encounter your enemy's ox or ass wandering, you must take it back (36). "If you see your fellow Israelite's ox or sheep gone astray, do not ignore it; you must take it back to your fellow. If your fellow Israelite does not live near you or you do not know who [the owner] is, you shall bring it home and it shall remain with you until your fellow claims it; then you shall give it back" (37, 38).

Beyond logic utilitarian and social implications as "saving a good" and "saving a fellow's good", we cannot ignore some animal welfare implications *ante litteram,* as in Rashi's comment about interpreting Shabbat's rest for animals not only like a mere abstention from work, but also as really "enjoying the day" and feed free on the pasture.

As previously mentioned, the explicit permission to feed on animals was given only after the Great Flood: "Every creature that lives shall be yours to eat; as with the green grasses, I give you all these" (19). However, with specific limitations and these refer at the time of Noah, which means before the Patriarchs and even before the concept itself of Jewish people or Israel, they have an intrinsic value for the entire humanity: "You must not, however, eat flesh with its life-blood in it" (38). Commentaries by the Sages are extremely interesting: the expression "its life-blood" implicates the prohibition to "the eating of a limb cut from a living animal" (40), which means the obligation to kill completely an animal before feeding on it. And, "It was prohibited to cut off a limb of a living animal and eat it, because such an act would produce cruelty" (41). "In truth, there is no greater cruelty in the world

then the one who cuts a limb or meat from an animal while it is still alive in front of him and eats it" (42).

Different commandments and comments dealing with feeding on animals underline the concept that, despite the fact that we are allowed to feed on them, this must be done in a way that the possible suffering caused to them will be minimal as possible (43, 44). Notwithstanding that sages anyway forbid consciously induced suffering.

Other obligations or commandments from the Torah, as they mentioned, do not seem to have a significance strictly linked to avoid animals' physical sufferings, or satisfy their basic needs (feeding, etc.). For this reason, comments by Sages are particularly interesting: Prohibition to slaughter very young livestock, below the 8th day from birth: "When an ox or a sheep or a goat is born, it shall stay seven days with its mother, and from the eighth day on it shall be acceptable as an offering" (45). "As before then, it is not fit for anything – and no man would desire it for eating or for commerce or for a gift" (46).

Prohibition to pick, in nature, eggs or chicks in the presence of the mother: "If, along the road, you chance upon a bird's nest, in any tree or on the ground, with fledglings or eggs and the mother sitting over the fledglings or on the eggs, do not take the mother together with her young.

Let the mother go, and take only the young, in order that you may fare well and have a long life" (47). "If the mother is left free, she does not grieve (48). "It is from the roots of this commandment to put into our hearts that the providence of God ….is upon all of His creatures - with the human species individually……and therefore, no species will ever become extinct from all of the species of creatures" (49). "In the matter of dispatching the mother bird before taking her chicks, we find some display of protective concern by the Torah for the preservation of the species, an effort not to destroy the seed of the birds of the field although they are "*hefker*" (*res nullius*), unclaimed property" (50). Again, these glosses and comments induce us to think about an "environmentalist thought" *ante-litteram.*

Is a non-physical suffering considered? Torah forbids slaughtering a mother (genitor) and offspring the same day. "However, no animal from the herd or from the flock shall be slaughtered on the same day with its young" (51). The Sages comment: "And we can also express about the matter from the angle of the simple understanding as well, that this is to fix in our souls the trait of compassion and to distance us from the trait of cruelty – which is a bad trait. Therefore even though God permitted us [to eat] species of animals for our sustenance, He [also] commanded us that we not kill it and its child on one day to fix the trait of compassion in our souls (52). "People should be restrained and prevented from killing the two together in such a manner that the young is slain in the sight of the mother; for the pain of the animals under such circumstances is very great. There is no difference in this case between the pain of man and the pain of other living beings, since the love and tenderness of the mother for her young ones is not produced by reasoning, but by imagination, and this faculty exists not only in man but also in most living beings" (53).

"If the Law provides that such grief should not be caused to cattle or birds, how much more careful must we be that we should not cause grief to our fellowmen" (41). Even if the Rambam admits that not only humans, but also animals may have feelings and sensations, as all living beings, nevertheless reason of the commandment was that inducing suffering has a negative influence on man itself, making him getting used to evil.

In the light of so many explanations and comments relative to the respect for animals, if Torah would consider it unfit or cruel, the shechita (Kosher Slaughter) would not have been considered as a slaughter method.

Shechita has been indicated by Torah as a compassionate instrument for killing of animals destined to food for human consumption. Biblical text, in a strict sense, does not precisely prescribe or dictate how it is allowed to slaughter an animal. The verse "you may slaughter any of the cattle or sheep that God gives you, "*as I have instructed you*"; and you may eat to your heart's content in your settlements" (55), was interpreted by Sages as a reference to the oral tradition, which dictates, in the way I have *orally* instructed you. This was codified as, it "teaches about cutting the gullet and about cutting the windpipe, and about the requirement to cut the majority of one *siman* (sign: either gullet or windpipe) for a bird, and the majority of two *simanim* (signs: both gullet and windpipe) for an animal (livestock) .... and the veins (in a bird) (55). "In livestock, also the cutting of the veins" (56), where "veins" are intended as blood vessels in general.

This manner of slaughter is completed to induce death in the animal avoiding unnecessary pain: "and we can also say as a reason for slaughter from the neck with a checked knife, [that it is] in order that we not cause too

much pain to living beings. As the Torah [only] permitted man – due to his status – to derive nourishment from them for all of his needs, but not to cause them pain for no reason" (57); which may be considered an animal welfare statement *ante-litteram* when we think was written in the XIII century.

Furthermore, it is useful to be reminded of the prohibition to castrate: "You shall have no such practices (mutilations) in your own land" (58), which prohibition is observed, at least in Israel, relative to livestock. Relatively to dogs and cats, some modern teachers allow spaying females, as it is considered a lighter violation of the commandment, like making males infertile through reduction or elimination of blood drain without removing the testicles; or allowing temporary measure (hormonal castration; ligation of *vas deferens* and/or tubes in females).

Urgent therapeutic needs do not fall under the prohibition; neutering done for the sake of the animal's health may be considered permitted and essential (59). Likewise, if the procedure is performed to spare suffering and distress in the animal, for example neutered dogs being less likely to leave home, which may spare the life of the dog from death by automobiles, hunger or other hazards.

The Chief Rabbinate, in the past, considered a more lenient approach for public safety concerns from wild and ownerless animals, in order to achieve control of the stray populations (dogs, cats) and having as final goal a collective benefit and/or prevention of diseases, even zoonotic; with recommendation on who should perform the procedure, and how.

The use of animals for work, and relative norms, are nowadays scarce or without implications. However same norms could find new interpretations according to different situations in which animals are kept or found in modern society. For example, the obligation to feed, including stray animals (cats), which once tolerated have become "fellow citizens" dependent on humans, for which the Supreme Court of Israel forbid their killing as a solution to the "simple nuisance" caused by their presence (60).

The obligation for (veterinary) care for livestock and pets, including diseases prevention through effective vaccinations (61); assistance at parturition; limitation in use for medical purposes; prohibition, in a wider sense, for cruel practices. For example, the prohibition to restrain and force feeding for geese destined to production of *foie – gras* (62). The prohibition to assist to "corrida" bullfights (63). The prohibition of hunting for sport or cruelty (64), and because of "useless (*the prey killed by hunting is not kosher*) and dangerous destruction of living beings" (65). The prohibition for traditional "white veal" rearing, because "even if we could take into account that human needs could justify a certain suffering in animals, certainly white veal rearing is for the benefit of a small population only; and this does not justify that type of suffering" (66). Indeed, improved conditions requested in Israel for white veal rearing, in fact reduced this kind of industry to almost zero; however, the same considerations are used for animal fur prohibition (66).

Male chicks from eggs-producing lineages are generally killed at hatch, together with ill/deformed chicks. Killing of ill/deformed chicks is aimed to prevent further suffering which it can be done in a way which ensures minimal suffering. The killing of male chicks has been considered acceptable while there was no way to sex embryos and prevent male chicks development in eggs-producing lineages; and it is done with as less suffering as possible. In such a perspective, six to eight million chicks are killed at their hatch every year in Israel; some 7 billion in the EU. A biomarker, identified in 2016, allows a fast and highly precise determination of the sex of an egg (>99%) in a very early stage (<12 days), before development of pain-sensitivity of chicks. The automated solution detects if an egg contains male or female chicks, which allows removing males from incubators early in the breeding process and only hatch female layer chickens (67). Another solution, based on genetic sexing of chromosomes, allows female embryo development only at an early stage, while leading to the expression of the embryonic lethality-inducing gene in males embryos, which will not develop, with an expected accuracy of 100% (68). From an animal protection point of view, these systems should definitely be taken advantage of.

THE MEANING OF ANIMALS-RELATED COMMANDMENTS

Nature exists, and it has its own laws. It is the duty of the Jew is to be compassionate towards the creation and nature, even if time to time its laws seem *ruthless* to us.

The commandments were not given to change the order of the creation, rather to elevate man over nature, and imbue man with something divine: the mercy of God himself in

relation to his creation and living beings: "God is good to all, and his mercy is upon all his works" (69).

Rav Yehudah suffered for a long period, because of not having showed compassion to a veal destined to slaughter (70). His suffering ceased the day he showed compassion towards a little mouse and her offspring found in his house…

Vegetarianism and Abstaining from Meat.

The Torah in general, does not demand abstaining from eating meat or from their products; on the other hand, the Torah has no requirement for eating meat every day, rather on particular occasions such as during the time of the presence of the Sanctuary in Jerusalem, like Pesach sacrifices and other festive occasions. On the other hand, the Sages understood how it is in man's nature to look for satisfaction also in food: "A man is obligated to gladden his children and the members of his household on a Festival"… Rav Yehudha Ben Batira says "when the Temple was standing, rejoicing was only through meat; and now that the Temple is not standing …..only by wine" (71).

Abstaining from, or diminishing the consumption of meat are considered much more important from a health point of view rather than a moral ideal (72). As such, even if decisive thinkers in modern and Zionist Judaism see in the framework of "vegetarianism and peace" that ideal world represented by the experience of first Man at the beginning of Creation (73), which means, before meat consumption by man.

The Torah does not forbid the killing of animals to use them for food; the Torah teaches us to sanctify with living beings and goods: "when you have eaten your fill, give thanks to your God for the good land given to you" (74). Vice-versa, the Torah forbids the waste of the creation, living beings and objects, and it forbids the destruction of the Creation.

Animal Welfare & Freedom of Religion: the *Vexata quaestio* of Shechita in Europe and North America.

In recent years, a significant dispute involved the compatibility of shechita (Kosher Slaughter) with European Union legislation. European Union adopted two relevant acts, Council Directive 93/119/EC of 22 December 1993 on the protection of animals at the time of slaughter or killing, which establishes common minimum rules for the protection of animals at the time of slaughter or killing in member States. The directive has been followed by Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. The 2009 Regulation states at Whereas, 20: "Many killing methods are painful for animals. Stunning is therefore necessary to induce a lack of consciousness and sensibility before, or at the same time as, the animals are killed. Measuring the lack of consciousness and sensibility of an animal is complex and needs to be performed under scientifically approved methodology. Monitoring through indicators, however, should be carried out to evaluate the efficiency of the procedure under practical conditions." And at Whereas, 21: "Monitoring stunning efficiency is mainly based on the evaluation of consciousness and sensibility of the animals. The consciousness of an animal is essentially its ability to feel emotions and control its voluntary mobility." Article 4 of the 2009 regulations prescribes stunning as a compulsory procedure in the slaughtering process, as stunning is deemed to reduce pain in the animal.

The European Union legislator, while protecting animal welfare, was fully aware of the religious requirements that may preside over animal's slaughter. In Protocol No. 33: "On protection and welfare of animals", of the European Union Treaty (then EC), it is recognized the interplay between animal welfare and religion, when affirming that the Community "shall pay full regard to the welfare requirements of animals, while respecting the legislative or administrative provisions and customs of the Member States relating in particular to religious rites, cultural traditions and regional heritage" (75). Regulation 1099/2009: Whereas,18, balances prescriptions on prior stunning with religious freedom, as it provided derogation of prior stunning in case of religious slaughter. The derogation was provided for in Article 4 (4), which reads: "In the case of animals subject to particular methods of slaughter prescribed by religious rites, the requirements of paragraph 1 [compulsory stunning] shall not apply provided that the slaughter takes place in a slaughterhouse." The exception to compulsory prior stunning for religious reasons, encompassed in the Regulation, therefore, "respects the freedom of religion and the right to manifest religion or belief in worship, teaching, practice and observance", as enshrined in Article 10 of the Charter of Fundamental Rights of the European Union. It includes an exception to compulsory stunning for religious rites (Whereas, 18). Regulation 1099/2009 at whereas, 43,

describes how slaughter should be conducted without stunning: "Slaughter without stunning requires an accurate cut of the throat with a sharp knife to minimize suffering. In addition, animals that are not mechanically restrained after the cut are likely to endure a slower bleeding process and, thereby, prolonged unnecessary suffering"; "therefore, ruminants slaughtered without stunning should be individually and mechanically restrained".

At the same time, the European legislator acknowledged how the exceptions to compulsory stunning for religious reasons, already granted by Directive 93/119/EC, have been transposed differently by Member States depending on national context. In Whereas, 18, the Regulation deems necessary on the subject of derogation from stunning animals prior to slaughter should be maintained a certain level of subsidiarity to each Member State. Growing political pressures, as well as a certain forms of animal rights activism among the European public opinion regarding animal welfare, brought this legislative base to the European Court of Justice (ECJ) analysis.

In 2018, ECJ decided a case where the Flemish Region challenged the validity of Article 4(4) of Regulation No. 1099/2009. The case originated from the denial of the Belgian Government to approve "temporary slaughterhouses", during the "Feast of Sacrifice", to satisfy the increase in demand for halal meat during this Feast. The Court confirmed that ritual slaughter without stunning might take place only in approved slaughterhouses. The Court, considered the Article 4(4) of Regulation 1099/2009, in the light of article 10 of the European Chart of fundamental rights. That obligation (individual, mechanical restrain, as above explained) did not infringe freedom of religion as it was only intended to organize and manage the freedom to practice ritual slaughter, taking into account the fundamental rules on the protection of animal welfare and the health of consumers of meat.

In 2020, Jewish and Muslim Belgian Organizations challenged in front of ECJ a Belgian regulation prohibiting the slaughtering of animals by means of traditional Jewish and Muslim rites, as the regulation required such animals should be stunned prior to slaughter in order to reduce their suffering where the regulation introduced the concept of "reversible stunning". The Court decided that Member States have the legislative and administrative power *to limit* the application of Article 4(4), but are not permitted *to prohibit* the slaughter of animals without stunning, that also applies to the slaughter carried for a religious rite. At the same time, Member States can require an alternative stunning procedure for the slaughter carried out in the context of a religious rite, based on reversible stunning and on condition that the stunning should not result in the death of the animal.

On the one hand, the Court wanted to affirm the right, worded in Article 10 of the Charter of Fundamental rights manifest one's religion, while, on the other hand, prohibits ritual slaughter in the name of animal welfare. The case has been severely criticized by scholars and Jewish organizations, which tried to show, as we exposed above, that shechita respects animal welfare (76, 77). We should note that in both cases, ECJ acknowledges the lack of similar requirements (stunning) in the context of "hunting and recreational fishing activities or during cultural or sporting events", and, surprisingly, retains this is "not contrary" to animal welfare and protection at time of killing (78, 79)! Judaism would never have considered "hunting or killing during sporting events" as a practice to be set apart from animal welfare considerations; in fact, Judaism forbids these activities because they definitely have an impact on animal welfare: a negative one, and a useless reason.

A completely different approach is given by North American Legislators, where the "Humane Methods of Slaughter Act", 1958 (reinforced on 1978) at §1902, while reinforcing the concept that "No method of slaughtering or handling in connection with slaughtering shall be deemed to comply with the public policy of the United States unless it is humane"(80). It clearly states: "Either of the following two methods of slaughtering and handling are hereby found to be humane:

(a) in the case of cattle, calves, horses, mules, sheep, swine, and other livestock, all animals are rendered insensible to pain by a single blow or gunshot or an electrical, chemical or other means that is rapid and effective, before being shackled, hoisted, thrown, cast, or cut; or

(b) by slaughtering in accordance with the ritual requirements of the Jewish faith or any other religious faith that prescribes a method of slaughter whereby the animal suffers loss of consciousness by anemia of the brain caused by the simultaneous and instantaneous severance of the carotid arteries with a sharp

Table 1: the "5 freedoms concepts" and references in Judaism dealing with same concepts.

The "5 freedoms" concept of 1965	References in Judaism
Freedom from hunger and thirst	Devarim – Deuteronomy 11:15
Freedom from discomfort	Shemot – Exodus 23:5 Devarim – Deuteronomy 22:10 – Ibn Ezra A., *gloss on* Devarim – Deuteronomy 22:10 Devarim – Deuteronomy 22:1, 2 Einger D., 2015 (white veal rearing)
Freedom from pain, injury, or disease	Shemot – Exodus 23:5 – Hizchoni, *gloss on* Shemot – Exodus 23:5
Freedom to express normal behavior	Shemot – Exodus 23:12 – Rashi, *gloss on* Shemot – Exodus 23:12 Rambam, More' Nevuchim, 3:48
Freedom from fear and distress	Shemot – Exodus 23:5 – Hizchoni, *gloss on* Shemot – Exodus 23:5
Further animal protections aspects considered in Judaism	
Compassionate slaughter	Bereshit – Genesis 9:4 – Rashi, *gloss on* Bereshit – Genesis 9:4 Rambam, More' Nevuchim, 3:48 HaLevi A., Sefer Ha Chinuch, ch. 452
Prohibition of enjoying/participating to animal suffering	Yossef O., Sheelot ve-teshuvot, 17/10/2010 (bullfighting)
Prohibition of hunting	Rambam, More' Nevuchim, 3:17 Landau Y., Noda B'Yehudha, on "Yore' De'a", question 10th.

instrument and handling in connection with such slaughtering" (80).

The different attitude of the two legal systems, EU and USA, reflects their cultural beliefs, which, we may argue, have little or nothing to do with animal welfare. As we have demonstrated, Judaism is particularly aware of animal welfare and ritual slaughter highly considers the need to avoid animal suffering. The decision to prohibit ritual slaughtering without stunning could be prompted by some desire of uniformity more than a real respect for religions and animals.

CONCLUSIONS

Definitely, Judaism has a position relative to animals:

- awareness they are living beings
- awareness they can suffer and not only physically
- awareness they have needs and necessities.

Living beings are integral part of the Creation, together with the environment.

These clear stances anticipated by many centuries of today's known concepts summarized under the title of "The 5 freedoms" (81), defined in UK on 1965 and summarized in Table 1. Of such an ancient anticipation, Jews should be only proud! Judaism teaches to exercise a compassionate dominion on animals, environment, so that we will become accustomed to respect and be compassionate towards our fellows. Table 1 below summarizes the "5 freedoms", 1965 and recalls the similarities in Judaism.

It is imperative to be compassionate towards animals; a possible Jewish legislation relative to animals (e.g farming, transportation, slaughtering, killing for public health reasons, etc.), at least in the State of Israel, should have as main pillars the teaching of our Sages:

- protecting animals from harmful or dangerous situations, minimizing any traumatic event;
- abstaining from any intentionally traumatic action, abstaining from any gratuitously painful action.

The "key" for understanding is the awareness use of the Creation: benevolence use of animals, with prohibitions to their suffering and prohibitions to wasting or destruction.

Note: Translations from Hebrew, Aramaic original texts are according to: https://www.sefaria.org/ *in order to guarantee uniformity of language.*

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The History of Highly-Pathogenic Avian Influenza in Israel (H5-subtypes): from 2006 to 2023

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ABSTRACT

Avian Influenza (AI) has become the largest animal epidemic in the world. So far hundreds of millions birds have died or been culled due to the disease. A novel highly-pathogenic avian influenza (HPAI) subtype H5N1 virus, which emerged in 1996 in domestic geese in China, passed from wild waterfowl as a low-pathogenic (LP) virus, and in the domestic birds was modified to a High Pathogenic (HP) virus due to mutational addition of basic amino acids to the cleavage site of the Hemagglutinin (HA) protein. This "A/goose/Guangdong/1/1996" virus killed poultry but also infected at least 18 people of whom 6 died. In 2003-6 the virus spread to Asia, Europe, Africa and the Middle East. Mass mortality of migratory birds at the Salt Qinghai Lake in West China in 2005 was a turning point in the understanding of the virus co-circulation between poultry and wild birds. Israel is situated on migratory routes for over a billion birds each year in the autumn migration from Europe to Africa and in the northwards spring migration, thus the potential for virus transmission is high. The 1996's parental virus emerged in Israel in 2006, following the Qinghai Lake event, and included meat turkeys, heavy breeders and broilers. Since then, several focal outbreaks occurred in Israel every 1-3 years. In 2012, the same AIV-H5N1 was detected in meat turkeys and found also in alley cats following consumption of the bird carcasses, with 100% identity of the HA gene. A second multi-focal outbreak of AIV-H5N1 appeared in 2015 following the worldwide wave in 2014-15. This outbreak included meat turkeys, heavy breeders and egg layers. These viruses belonged phylogenetically to clade 2.2.1.2, emerged from previous clades 2.2 and 2.2.1.1. Reassortment of viral segments of a descendant-H5 AIV of the Chinese parental virus with AIV-N8 viruses created novel H5N8 viruses, first detected in China in 2010. An 2016's variant of this virus (group-B Gochang-like, clade 2.3.4.4), was discovered in May-June 2016 in wild swans at Lake Ubsu-Nur on the Russian-Mongolian border, and reached Central Asia, Europe, Africa, Middle East and Israel. The Ubsu-Nur 2016's AIV-H5N8 was genetically identical to an AIV-H5N8 that emerged in Israel six months later during the autumn migration. This was the first H5N8 outbreak in Israel, which included meat and breeder turkeys, heavy and light breeders, layers, breeder ducks and backyard poultry, but also many wild birds, not seen in the previous H5N1 outbreaks, of at least 16 species mostly waterfowl and raptors. The cleavage site of HA gene showed similarity between poultry and wild birds, as well as to grp B-2.3.4.4 European-Asian H5N8 viruses. Since October 2020, new H5 reassortants with LPAI viruses from wild birds contributed the Neuraminidase (NA) glycoprotein, have appeared in Europe and created new H5 strains. A second global outbreak of a new H5 variant occurred in 2020-21 in Russia and the Palearctic region. Following this global outbreak, a second AIV-H5N8 outbreak appeared in Israel in 2020 during the autumn migration. The outbreak included meat and breeder turkeys, heavy breeders, broilers, game birds mainly black swans (*Cygnus atratus*), and wild birds. The 2021–22's AIV-H5N1 epidemic season was the largest so far in Europe and Eurasia, with about 2500 outbreaks in poultry and culling of 50 million birds, and about 3600 detections in wild birds. That outbreak followed the 2020's wave of the new AIV-H5N8.

Following this epidemic, a third multi-focal outbreak of AIV-H5N1 occurred in Israel in 2021, in meat and breeder turkeys, heavy breeders, organic egg layers and meat ducks. The 2021's outbreak was characterized also by affecting many wild birds, not seen before in H5N1's outbreaks in Israel, while the largest affected population was Common cranes (*Grus grus*) on their migration routes from Russia and Scandinavia to Ethiopia and Sudan through Israel, undergoing mass mortality of almost 10,000 birds. Other threatened species like Marbled teal (*Marmaronetta angustirostris*) have died also due to this virus, as well as hundreds of Great white pelicans (*Pelecanus onocrotalus*), and several species of waterfowl and raptors. Classification of the viruses by whole genome sequencing (WGS) revealed the same group B-2.3.4.4 clade as in the H5N8 events and similarity between poultry and wild birds. This virus probably came from Russia in the autumn migration. A new outbreak of AIV-H5N1 began emerging in Israel from November 2022, in meat turkeys, heavy and light breeders, broilers. Unlike the 2021's outbreak, this virus affected only a few wild birds. HA gene sequencing revealed the virus belonged to the same group B-2.3.4.4 clade as in previous outbreaks. In conclusion, all avian influenza subtype H5 outbreaks in Israel followed the European-Eurasian outbreaks. The virus origin was from South East Asia, reaching Israel through spillover with migrating birds via Eurasia and Europe.

Keywords: Avian Influenza; AIV-H5N1; AIV-H5N8; Clade; Spillover.

INTRODUCTION

Avian Influenza has become the largest animal epidemic in the world, until today hundreds of millions of birds have died or been culled due this infection.

Avian Influenza Virus (AIV) is an 8-segmented *Ortomyxoviridae* type A enveloped single-stranded RNA virus with a size ranging from 80 to 120 ηm. The virus contains two important envelop glycoproteins that determine its classification into subtypes, one of 18 different Haemagglutinin (HA) proteins and one of 11 different Neuraminidase (NA) proteins. However birds have one of 16 different HA's and one of 9 different NA's. Wild birds are natural reservoir for the 16 HA and the 9 NA subtypes of AIV. The HA protein must undergo proteolytic cleavage into two subunits to allow conformational changes required for membrane fusion and subsequently the virus pathogenesis process. According the number of basic amino acids (lysine, arginine) in its cleavage site (a part of the HA protein) the virus may be defined as low-pathogenic (LP, 1 or 2 basic amino acids) or high-pathogenic (HP, more than 2 amino acids). Only H5 and H7 AIV's are defined as highly-virulent viruses. AIV-HPAI's originated in China as a mutation from AIV-LPAI's that were introduced from wild birds to farm birds – and multiplied in the latter, mainly due to RNA-polymerase errors that caused the addition of basic amino acids to the cleavage site or the replacement of non-basic amino acids with basic ones, but also changes in other genes (1, 2).

The most quoted date for the beginning of Avian Influenza (AI) in poultry is 1878 when a sudden plague-like disease in poultry appeared in Italy, initially known as "fowl plague" confused with acute septicemic fowl cholera but later renamed to "highly pathogenic avian influenza", given by the Italian veterinarian Edoardo Perroncito (3). A defined AIV-H5N1 virus was isolated for the first time in 1959 from chickens in Scotland (4), AIV-H5N3 in 1961 from common terns in South Africa (5), and a list of many other HPAI viruses from poultry, including H5 and H7 subtypes (6). All these were sporadic outbreaks in commercial or backyard poultry and non-pathogenic for humans. Outbreaks of HPAI's viruses until 1995 caused only minimal losses (7), while the eruption of the H5 outbreaks led to the current waves which began in 1996.

Highly-pathogenic H5N1 emerged in 1996 as a novel strain in domestic geese in Guangdong, Southern China, that passed from wild waterfowl, while in the domestic birds the virus was modified by a mutation to become a highly-pathogenic virus (by addition of basic amino acids

DEDICATION:

This article is dedicated in memory of Prof. Yorham Weisman, Dr. Shimon Perk and Dr. Ezra Rosenbluth. all of whom mentioned played an important role in the research concerning Avian Influenza.

to the cleavage site). This novel virus killed in 1997 over 3 million chickens in Hong Kong, and then, for the first time in humans, infected at least 18 people in Hong Kong, 6 of them died (the epidemic in humans was called "Hong Kong Influenza") (8). Therefore, all bird populations on the island were culled to disable the spread of the virus. This progenitor virus was called "A/goose/Guangdong/1/1996" (9).

In 2003-6 the virus spread in Asia, Europe, Africa and the Middle East, and burst into the public mind as dangerous to birds and to humans. The turning point happened in 2005 when thousands of migratory birds died due to HPAI at one of the main migration stations in western China, the salt lake called "Qinghai Lake". Mass mortality of migratory birds at the salt Qinghai Lake in West China in 2005 was a turning point in the understanding of the virus co-circulation between poultry and wild birds (10).

Since 2003 until 6 January 2023, WHO reported 868 cases of human infection by AIV-H5N1 from 21 countries of which 457 individuals died (Case Fatality Rate, CFR of 53%) (11), with another two cases reported in February 2023 in Cambodia, one of them was fatal (12). Transmission between countries has been largely attributed to migratory wild birds. Most of the human deaths were in Egypt, Indonesia and Vietnam (11).

In the last two years, 2021-22, AIV affected most European countries and is considered there endemic. Israel is situated on migratory routes of more than 500 million birds twice a year, in the autumn migration from Europe to Africa and in the spring migration northwards (13). As most of the migratory birds arrive from AIV-infected countries, and some stay to winter in Israel, the potential for transmission of the virus from wild birds in Israel is high.

The HP AIV-H5N1 virus has a short incubation period in the affected birds, with a huge swift mortality within 24-48 hours, leading to very high economical losses. The virus may affect all types of domestic and captive birds, although wild waterfowl may serve as subclinical carriers (1). The characteristic clinical signs in commercial poultry include sudden onset of severe illness along with rapid mortality, up to 100% in a few days, even 1-2 days, without apparent signs. When signs do appear they include depression, ruffled feathers, severe respiratory distress, coughing, sneezing, eye inflammation or conjunctivitis, sinusitis, cyanosis of the head, combs and wattles, swelling/edema of the head, face and sinuses, diarrhea, nervous signs, and in breeders and layers a drop or cessation of egg production (1).

According reports from 2005 onwards, the virus may be also fatal and kill these subclinical carrier birds (14, 15), however sporadic deaths of wild waterfowl and other migratory birds due to ancient AI viruses were reported even years before (6). The virus is spread by respiration, nasal and mouth discharge, droppings or close contact with infected birds. Humans can also be infected through bird slaughter, feather plucking, cleaning and preparation of poultry meat for cooking, eating raw meat and contact with contaminated blood, for example by sucking contaminated blood of poultry as customary in eastern countries like Japan, Thailand, etc (16). Elevated concern of mutations by gene transfer between influenza viruses, changes in receptor affinity (antigenic shift), and the emergence of a novel pandemic virus that has the feature of man-to-man infection always exists.

Post-mortem lesions of HP AIV's include mainly hemorrhages, edema and necrosis of multiple organs along with inflammation, including skin of the comb or wattles, legs and toes. Most severely affected organs are liver, pancreas, heart, lungs, kidneys and brain. Due to the hyper-acute nature of the disease, it is possible that none of the mentioned lesions will be seen (1).

Another highly-pathogenic reassortment of H5 with HxN8 viruses, namely H5N8, was first detected in a live poultry market in China in 2010, then in South Korea and Japan. The hemagglutinin coding segment of H5N8 was a descendant of the Chinese parent virus – 1996 HPAI H5N1 (A/goose/Guangdong/1/1996).

In fact, the first known outbreak of AIV-H5N8 subtype occurred in poultry in Ireland in 1983 (A/duck/Ireland/113/1983, A/turkey/Ireland/1378/1983), over 300,000 domestic ducks, chickens and turkeys were culled where turkeys were the most susceptible bird (6). Twenty years later the virus was reported in US (A/avian/New York/Sg-00418/2003). These viruses were different from the current 21st century viruses.

A 2014 variant of H5N8 appeared for the first time in South Korea as an outbreak in breeding ducks, other poultry and wild birds. The variant reached China, Japan, Europe and North America (group-A Buan-like, clade 2.3.4.4), but did not reach Israel (17, 18, 19). The virus was introduced into Europe probably via the autumn migration of wild birds returning from Russia and Asia with routes going from

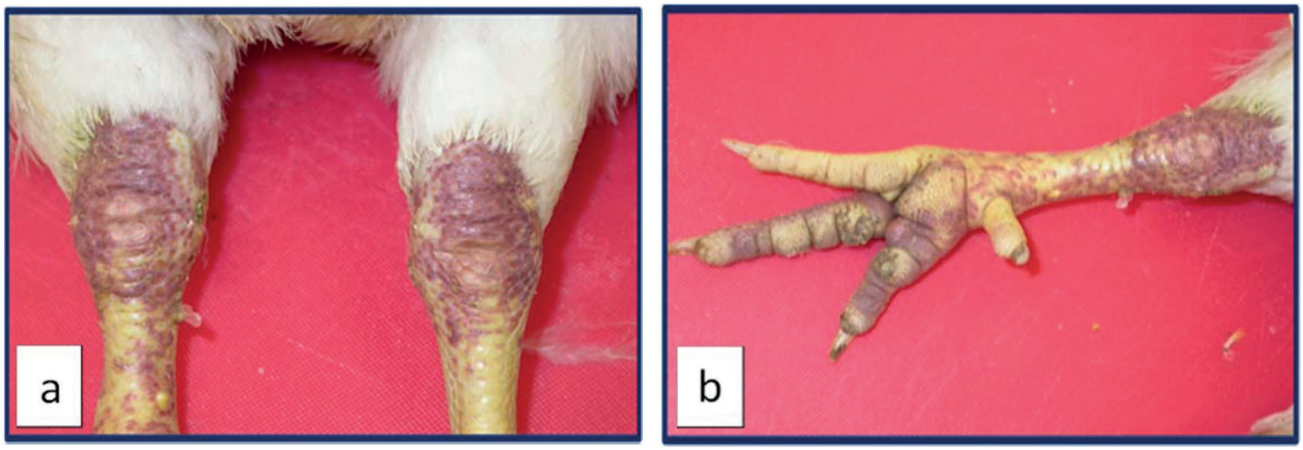

Figure1. Focal hemorrhages in legs (a) and toes (b) skin of heavy breeders infected with AIV-H5N1 (Ein Shemer, 2010, photos by Dr. A. Lublin, Kimron Veterinary Institute, Bet Dagan).

northeast to southwest. However, a 2016's variant of this virus (group-B Gochang-like), reported in South Korea and Taiwan in domestic and wild birds, discovered in wild swans in Lake Ubsu-Nur in Tyva Republic on the border of Russia-Mongolia in May-June 2016, reached Central Asia, Europe and the Middle East including Israel and Africa. Group-B variant belongs to the same 2.3.4.4 clade. Clade 2.3.4.4 was found to reassort with 6 different neuraminidase genes. As different H5Nx viruses may circulate at the same time in the same area, the contribution of the migratory birds was clear.

Clinical signs in birds of this variant virus include apathy, incoordination, seizures, recumbency and mortality. Unlike AIV-H5N1, AIV-H5N8 variants it is less virulent to humans. A virus of the H5N8 strain caused mild and limited or asymptomatic disease in seven poultry workers in south of Russia in an outbreak of AIV-H5N8 on a poultry farm (February 2021), without person-to-person transmission (20).

THE HISTORY OF VIRULENT AVIAN INFLUENZA VIRUSES IN ISRAEL.

Before the AIV-H5N1 eruption.

Virulent AIV viruses in Israel are known since the 70-80's of the previous century concurrently in domestic and wild birds. They include outbreaks of AIV-H7N2 (HP) in chickens (broilers) in Degania presenting as a respiratory disease, in breeder turkeys in Ramon and in duck farms. Concurrently, AIV disease was found in feral mallards, rock partridges and starlings (21, 22, 23, 24, 25, 26). In the turkey farms, the AIV infection occurred about 2 months after mortality of about 200 wild mallards that were found in close proximity to the turkey premises. Up to 80% mortality was found in the turkeys and a sharp drop in egg laying in the chickens, from 50-70% to 5-10%. The diagnostic tools at that time were serology and virus isolation (27, 28). The first isolation of avian influenza virus in Israel was in migratory starlings (*Sturnus vulgaris*) however it was a non-pathogenic (NP) virus (subtype H7N7) (21, 29).

AIV-H5 WAVES OF OUTBREAKS IN ISRAEL.

2006's multi-focal outbreak (H5N1). The 1996's Guangdong virus appeared in Israel for the first time in March 2006, following the Chinese Qinghai Lake event, and was identical to Egyptian strains. The first reported case of AIV-H5N1 in the outbreak in the Gaza Strip was 5 days later, and in Jordan, 7 days later. The first reported case of HPAI-H5N1 in Egypt was one month before the initiation of the outbreak in Israel. This outbreak included nine events in the Gaza Envelope area and in the Jerusalem area: six meat turkey farms, two heavy breeder farms and one broiler farm, and lasted 2 weeks. The maximal daily mortality was 35% (30, 31).

2008-2012's spot-separated outbreaks (H5N1). Since the 2006's multi-focal outbreak of AIV-H5N1, several focal distinct outbreaks occurred in Israel averagely every 1-3 years. These included: a back-yard chicken in 2008, heavy breeders in 2010 (focal hemorrhages in legs and toes skin are presented in Figure 1), and an Emu in a petting zoo, 3 flocks of meat turkeys in 2011 and one of them in the

Figure 2. A cat infected by AIV-H5N1 after consumption of carcasses of turkeys infected by this virus, displaying eye inflammation (photo by the courtesy of Dr. M. Bellaich, Kimron Veterinary Institute, Bet Dagan).

Palestinian Authority. In addition, in 2011 a Marsh Harrier (*Circus aeruginosus*) that was found ill in Jordan Valley and died later was found positive for AIV-H5N1 presence.

In 2012, two meat turkey flocks were found positive to AIV-H5N1. An outbreak of the same H5N1 virus was detected in alley cats following consumption of infected turkey carcasses from one of the flocks (32). The cats were seen eating sections of carcasses of the dead or euthanized turkeys. Clinical signs in the cats occurred several days later, mainly respiratory, dyspnea, ocular or nasal discharge and weakness, up to their acute death. The nucleotide sequences of HA genes of viruses isolated from the turkeys (ty/Israel/289/2012) and from the cats (cat/Israel/387/2012) showed 100% similarity, with 1.7% difference from the other turkey flock examined. One of these cats is presented in Figure 2.

2015's multi-focal outbreak (H5N1). The second multi-focal outbreak of AIV-H5N1 occurred in January 2015 following a worldwide wave of outbreaks in 2014-15. This outbreak lasted almost one month, in eight flocks, six meat turkey flocks and two heavy breeder flocks, all from the North-Ha'Sharon region. A Spur-winged Lapwing (*Vanellus spinosus*) found pecking at the manure of a turkey flock which had been euthanized. The Spur-winged Lapwing bird presented with clinical signs of AIV and died, and was diagnosed with AIV-H5N1. This outbreak included also four flocks from the Palestinian Authority, one of meat turkeys and three egg layers, from Jenin, Qalqiliya and Jerusalem districts. These flocks extended the outbreak period by another 6 weeks. The Laboratory of Avian Diseases at Kimron Veterinary Institute, Bet Dagan, assisted in testing some of the samples from the Gaza Strip, found 55 positive farms (most of the tested farms), most of them ducks and egg layers. Another focal event occurred 4 months later (in May 2015) in two meat turkey farms in the North of Israel, on the Lebanese border (33).

The AIV-H5N1 isolates belonged phylogenetically to clade 2.2.1.2 according to WHO nomenclature, emerging from previous clades, 2.2 in 2006's outbreak and 2.2.1 and 2.2.1.1.a in 2010-2012's focal outbreaks.

Figure 3 presents the full HA nucleotide sequences of AIV-H5N1 outbreaks – 2006, 2008-2012 (intermediate period between multi-focal outbreaks) and 2015, compared to global (mainly Egyptian) isolates. There is less than 0.5% difference in the sequences of the various isolates within 2015's outbreak, 3-4% difference between 2015's and 2008-2012's viruses, and about 7% difference between 2015's and 2006's outbreak (which resembles the sequence of the Chinese progenitor virus). As seen in Figure 3, some of the viruses of poultry in Israel are phylogenetically identical to viruses from ducks and chickens from Egypt and the Gaza Strip and the others are almost identical.

2016's multi-focal outbreak (H5N8). The first AIV-H5N8 virus appeared in Israel in November 2016 and lasted until February 2017. This outbreak in commercial birds included mainly meat turkeys but also breeder turkeys, heavy and light breeders, egg layers, breeder ducks and backyard poultry. In addition, many wild birds, not seen in the previous H5N1 outbreaks were affected. The affected wild birds included at least 16 species from 8 orders, mostly waterfowl and other water-inhabiting birds, and birds of prey (34).

The AIV-H5N8 diagnosed in late May–early June 2016 in wild birds in the Ubsu-Nur Lake, was genetically very similar to the virus that appeared in Israel six months later during the autumn migration to Africa, passing through Israel. Analysis of nucleotide sequences of the cleavage site of HA gene showed similarity of the isolates among them-

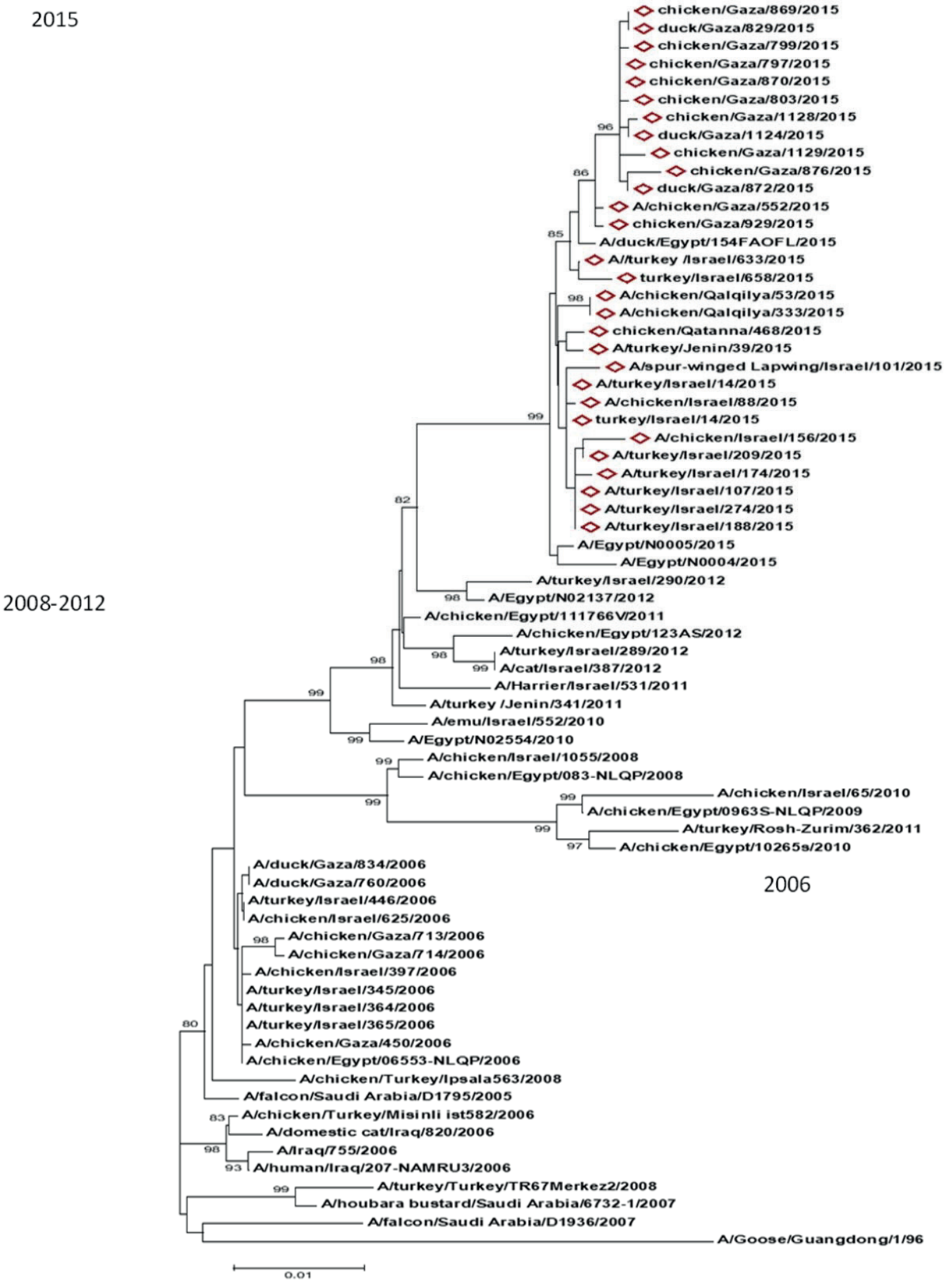

Figure 3. Full HA nucleotide sequences of AIV-H5N1 outbreaks – 2006, 2008-2012 (intermediate period between multi-focal outbreaks) and 2015, compared to global isolates.

Figure 4. HA-2 moiety nucleotide sequences of 2016's AIV-H5N8 outbreak compared to global isolates (Israel's viruses are presented in blue color).

Figure 5. Black swan (*Cygnus atratus*).

selves, with very minor differences, and similarity to H5N8 viruses from other parts of the world (in Asia and Europe), and belonging to clade 2.3.4.4 group B as these viruses (see Figure 4). A single case of AIV-H5N8 positive bird was discovered in January 2018 in a Eurasian eagle-owl (*Bubo bubo*) in Jerusalem.

Figure 4 presents HA-2 moiety (~900 bp out of HA total ~1800 bp) nucleotide sequences of 2016's AIV-H5N8 outbreak compared to global isolates. The Israel viruses belong to clade 2.3.4.4 group B (Gochang-like) as the Chinese and the European viruses, but were different from the clade 2.3.4.4 group A (Buan-like) of 2014's outbreak that had not reached Israel. As seen in the figure, the differences from the global viruses are minor but a sub-group of the Israel viruses was almost 2% different from most of the viruses.

2020's multi-focal outbreak (H5N8). Another global outbreak of a new H5 variant appeared in 2020-2021 in a farm and in wild birds in Russia and the entire Palearctic region. Since October 2020, new H5Nx reassortants have appeared in Europe on the basis of AIV-H5N8 clade 2.3.4.4B that contributed the HA glycoprotein, while LPAI viruses from wild birds in Asia and Europe contributed the NA glycoprotein, thus creating new strains of H5N1, H5N2, H5N3, H5N4, H5N5 and H5N8 (35).

A second AIV-H5N8 outbreak in Israel appeared in October 2020 during the autumn migration season, following the outbreak worldwide. Few months before, in the summer of 2020, there was an outbreak in Hungary, a country on the axis of crane migration from Scandinavia to Israel, also in Russia from which another migration route originates. This outbreak in Israel lasted for 3 months. The affected poultry branches were meat turkeys, breeder turkeys, heavy breeders and broilers.

This outbreak included game birds in several zoological parks and zoos mainly black swans (*Cygnus atratus*) (Figure 5) but also few a wild birds (Great white pelican, Eurasian eagle owl, Northern shoveler).

2021's multi-focal outbreak (H5N1).

From late 2021 to 2022, a new H5N1 virus causing poultry outbreaks worldwide belonging to clade 2.3.4.4B with a wild bird adapted N1 gene emerged. Those Clade 2.3.4.4B AIV-H5N1 European and Eurasian viruses followed the 2020 wave of a new H5N8 that contributed the hemagglutinin H5 to several reassortants with NA, including N1. The new AIV-H5N1 viruses became predominant in Asia, Africa, Europe, and the Middle East by the end of 2021 (36, 37).

The 2021–2022 the highly pathogenic avian influenza (HPAI) epidemic season was the largest HPAI epidemic so far observed in Europe. There was a total of about 2500 outbreaks in poultry with more than 50 million birds culled in the affected establishments. About 200 outbreaks were identified in captive birds. About 3600 HPAI virus detections were made in wild birds with an unprecedent geographical reach extent from Svalbard Islands to South Portugal and Ukraine, affecting 37 European countries (38).

Following the large HPAI epidemic in Europe, a third multi-focal outbreak of AIV-H5N1 occurred in October

Figure 6. Common cranes (*Grus grus*).

2021, first in a meat turkey farm in Northern Israel. The 2021's outbreak lasted until February 2022 and included over 10 meat turkey farms, one farm of turkey breeders, three heavy breeder farms, several farms of organic egg layers and a meat duck farm. The biggest outbreak was in an aggregate of over 50 premises of egg layers in a Moshav from North Israel, supplying almost 10% of the monthly supply of table eggs. Unfortunately, over 550,000 layers were culled. At least 85% of the affected poultry farms were close to large bodies of water with abundant wild birds, especially waterfowl.

The 2021's outbreak was characterized by a large number of wild birds, not seen before in AIV-H5N1's outbreaks in Israel, while the largest population was that of Common crane (*Grus grus*) (Figure 6) in their migration route from Russia and Scandinavia to North-East Africa, Ethiopia and Sudan, through Israel. The affected crane population underwent mass mortality of almost 10,000 birds in Agamon Hula, a part of the Hula Valley, a large water lake on the route of Jordan River in North-East Israel, but also in several other locations. Action was focused on disposing the carcasses, monitoring the spread of the outbreak, and reducing the large amounts of food (grains) spread to the cranes to minimize their damage to the fish raised in nearby ponds. Also, globally threatened species such as Marbled teal (*Marmaronetta angustirostris*) died, as have hundreds of Great white pelicans (*Pelecanus onocrotalus*) in several locations in Israel especially water reservoirs. Pelicans migrate from Russia to East and Central Africa while passing through Israel, however some of these birds stay to winter in Israel. Several other wild birds submitted to the laboratory of Avian Diseases at KVI and were found H5N1-positive, these included several species of waterfowl, several of raptors, and a hooded crow.

A genomic classification by whole genome sequencing (WGS) of about 45 isolates of poultry and wild birds (carried out by Prof. Michal Mandelboim and Dr. Neta Zuckerman, the Central Virology Laboratory, Sheba Medical Centre, Ramat-Gan), revealed in all of them the 2.3.4.4B clade, the same hemagglutinin as in the H5N8 events but different from the previous 2015's H5N1 outbreak (2.2.1). It was not possible to differentiate between the genomes of poultry and those of wild birds' viruses.

Figure 7 presents a HA-Phylogenetic tree of 2020-2021's AIV-H5N1 and AIV-H5N8 outbreaks, 2016's AIV-H5N8 (Israeli and global isolates), and another global AIV subtype (H5N6). As seen in the figure, the sequence of the first affected poultry farm in Israel (brown-colored) is identical to those of poultry and wild birds from Russia and Italy (marked in the figure). The H5N8 Israeli isolates are green-colored. The difference between the two H5N8's Israel outbreaks reached 1-2%.

Figure 8 displays pancreatic hemorrhages in a Common crane (*Grus grus*) affected with AIV-H5N1 (A) and in a Black swan (*Cygnus atratus*) affected with AIV-H5N8 (B). Pancreatic and splenic necrosis were reported as a common pathological findings in both Galliformes and Anseriformes infected by AIV-H5N1 in the United Kingdom (39). The pathological presentation of the lesions in both birds are similar.

Figure 7. HA-Phylogenetic tree of 2021's H5N1-AIV and 2020-2021's H5N8-AIV outbreaks (Israel H5N1 virus is brown-colored, and H5N8 viruses are green-colored), 2016's H5N8-AIV (Israeli and global isolates, Israeli isolates are green-colored), and other global AIV subtypes (H5N1, H5N6, red-colored).

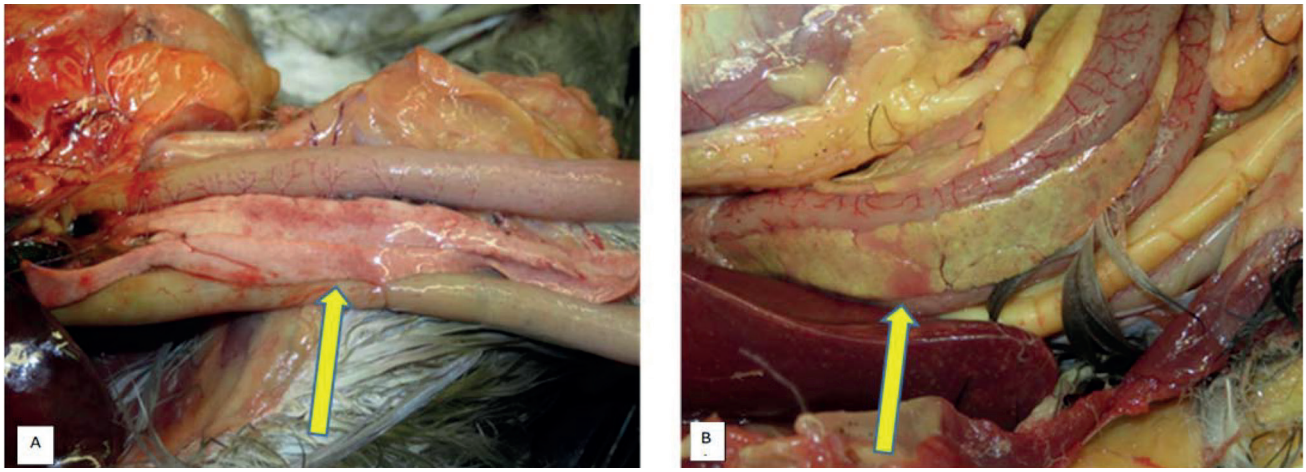

Figure 8. Pancreatic hemorrhages (marked by arrows) in a Common crane (*Grus grus*) affected with AIV-H5N1 (A) and in a Black swan (*Cygnus atratus*) affected with AIV-H5N8 (B) (photos by Dr. A. Berkowitz, Kimron Veterinary Institute, Bet Dagan).

2022's multi-focal outbreak (H5N1). According to all predictions based on the H5N1 outbreak in Europe and the migratory birds suspected of carrying the virus to Israel, a new outbreak of AIV-H5N1 indeed appeared in Israel since November 2022, starting in a meat turkeys flock, and lasted until January 2023. The outbreak included six meat turkey farms, two heavy breeder farms, one light breeder farm and two broiler farms. The appearance of AIV-H5N1 in broiler farms is uncommon and has almost never occurred in Israel in the past, possibly because of the young age of those birds. Unlike in the previous outbreaks, only three wild birds out of many which were tested, were found positive for AIV-H5N1.

DISCUSSION AND SUMMARY

Since the original A/goose/Guangdong/1996 H5N1 virus the HPAI-subtype H5N1 virus was detected and isolated from domestic poultry as well as wild birds in Asia, Europe and Africa, causing sporadic infections in humans and raised pandemic concerns. The virus is continuously co-circulating between domestic and wild birds and presents remarkable genetic diversity due to accumulating point mutations since the low pathogenicity AI viruses of H5 subtype were introduced into poultry. Initially, the importance of birds in the transmission of the virus between continents and countries was doubtful, but over time it has become clear that they are a very significant factor. It is accepted that both wild birds and domestic poultry participate in mutual transitions of AI viruses and their spread between continents and countries and within countries (7).

So far, AIV virus-H5 types have affected 66 countries, causing loss of hundreds of millions of birds (40). The virus entered into Israel through migratory birds and has spread to commercial poultry houses of all poultry sectors. Subtypes H5N1 and H5N8 of AI viruses replace each other along the timeline through common HA-5 glycoprotein and reassortment with other strains that provide the NA glycoprotein. All outbreaks in Israel emerged following Eurasian outbreaks and originated from these viruses and therefore the viruses isolated in Israel are similar or even identical to the European viruses.

Israel has experienced six AIV-H5-subtypes outbreaks (H5N1 and H5N8) in the years 2006-2023. All the outbreaks in Israel appeared following outbreaks in Eurasia and Europe. The first 2006's outbreak appeared after the Chinese Qinghai Lake event in 2005 (10). In that outbreak very few infected migratory birds were detected out of thousands tested and it can be assumed that after the initial entry of the virus, it underwent a secondary spread to various poultry farms in Israel probably through the movement of people and vehicles that may have transferred contaminated feces from infected to susceptible birds. According Balicer *et al* (41), several epidemiologic links between outbreak foci were identified, they and the near-simultaneous detection of several outbreak foci, increase the likelihood that the virus disseminated through use of shared vehicles or by personnel. Still, they did not rule out involvement of migratory birds in disease transmission. As can be seen in Figure 3, the viruses of poultry in Israel are identical or almost identical to viruses

from ducks and chickens from Egypt and Gaza Strip. The Egyptian researchers reported on outbreaks in commercial and domestic poultry in Egypt since February 2006 (42), one month before the first case in Israel in March 2006. The first affected farms in Israel were in the Gaza Envelope, leading to the assumption that the outbreak began following the introduction of the virus from the Gaza Strip.

Since 2014, HPAI clade 2.3.4.4 viruses have spread rapidly via migratory wild aquatic birds and have evolved through reassortment with prevailing local LPAI viruses (43). The 2015's outbreak appeared following outbreaks in Europe in 2014-15. In this outbreak similarly, only very few wild birds were found infected with the AI virus. The 2015's outbreak viruses were found to be also very close to viruses from ducks and chickens in Gaza strip and Egypt, and the sequences of HA were dissimilar from the 2006's AI viruses by 3-4% (Figure 3).

The 2016's AIV-H5N8 isolates are almost identical to European viruses such as from Poland, France and Croatia (Figure 4), and followed the global evolution of the virus, that is the appearance of new viruses such as H5N8 through reassortment. This was the first outbreak in Israel in which many wild birds were found to be infected with the virus. The similarity of the sequences of wild birds as well as domestic birds to sequences of European birds (Figure 4) is proof that the AI viruses were introduced from Europe through the seasonal migration of birds.

Since October 2020, new H5Nx reassortants have appeared in Europe on the basis of AIV-H5N8 clade 2.3.4.4B (34). Following outbreaks in Russia and other European countries in the summer of 2020, a second wave of AIV-H5N8 appeared in Israel several months later in the autumn migration of wild birds to Israel. The HA sequences of the birds in Israel were similar to those of the European and Russian viruses, as can be seen in Figure 7.

Comparing the AIV-H5N1 sequences of the 2021's outbreak in Israel, following the global 2020-21's outbreak (38, 44) to those of Europe, for example, poultry in Israel to poultry and wild birds in Italy and Russia as can be seen in Figure 7, is evidence to the introduction of the virus from Europe to Israel in the autumn migration. According sequences comparison carried out in the Laboratory of Avian Diseases at KVI, same H5N1 virus found about six months before in Africa, probably returned to Europe and Russia in the spring migration before its introduction to Israel in the successive 2021's autumn migration. As can be seen in Figures 4 and 7, since the 2016's outbreak in Israel, domestic and wild birds have almost the same H5 HA sequences. In those outbreaks, many wild birds, some of which were positive for AIV-H5, were found in close proximity to poultry that were also positive for AIV-H5. These findings may be an indication of the importance of wild birds in the transmission of the virus to commercial poultry in different locations in Israel.

The most severely affected bird population in Israel at this outbreak (global 2020-21's outbreak) was that of Common cranes (*Grus grus*) in their migration route from Russia and Scandinavia to North-East Africa countries through Israel (45). These big birds underwent mass mortality of almost 10,000 individuals in Agamon Hula National Park, one of the largest gathering at the route of their migration to Africa. Some of the birds remain in Israel for the winter, while most of them fly to Ethiopia, Sudan or farther as their final wintering destinations. Combat against further spread of the virus was focused on disposing the carcasses, monitoring the spread of the outbreak, monitoring presence of viral particles in water, and reducing the large amounts of food (grains) given to the cranes in order to reduce their damage to the fish raised in nearby ponds. Since the migration to Israel lasts about 3 weeks, it can be assumed that the cranes became infected in Israel or at a nearby destination on the way, probably from wild waterfowl that were clinically healthy but carry the virus.

To sum up the entire period since 2006 in which a highly pathogenic avian influenza H5-subtypes has been appearing in Israel every few years, it can be said that Israel is a portion of the global outbreaks especially those of Eurasia and Europe (46). The origin of these viruses is South East Asia and the transmission spillover is through migratory wild birds, mainly aquatic birds. Avian influenza outbreaks are integrated into the dynamics of the migratory seasons of the birds. Israel is located on migration routes and is therefore exposed to these viruses and to the phylogenetic evolution they undergo in the southward route from Europe to Africa and the northward route from Africa to Europe (13). It can be assumed that with the introduction of influenza viruses through wild birds during a new outbreak, the viruses spread in Israel reaches commercial poultry and zoos or animal parks raising ornamental birds through the migratory birds or the movement of resident birds. However, in some of the events,

spillover through people, poultry trading, vehicles and other mobile means may also have contributed to the spread of the virus between poultry premises in different settlements in Israel.

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Fowlpox Vaccination by Subcutaneous Injection, as an Alternative to the Wing Web Stab Application in Large Commercial Poultry Farms. A Field Clinical Study-Israel

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ABSTRACT

Fowlpox (FP) is an enzootic viral disease that infects domestic and wild birds globally. Although commercial poultry flocks have been vaccinated since the beginning of the last century, reports of outbreaks of the disease in vaccinated flocks have been accumulating, mainly in recent years. There are several hypotheses regarding the causes of fowl pox vaccine failure, but no re-examination of the process of vaccination in today's commercial flocks has been reported. After several cases of vaccination failure in vaccinated flocks, the authors of the article identified a critical problem with the conventional wing web (WW) stab vaccination method. A primary feasibility study was followed by a series of controlled field studies comparing the safety and efficacy of the traditional vaccination method of WW stab to the subcutaneous injection. The subcutaneous application was tested using a one dose per bird in different volumes. The studies were carried out in commercial rearing farms for layers and heavy breeders in Israel. The controlled field studies under commercial conditions included more than 20,000 birds. The results demonstrated that vaccination of pox vaccine by subcutaneous injection is completely safe, much faster, and more reliable than the WW stab vaccination method, providing a better and uniform immune response and protection and should be considered as an optional method for Fowlpox vaccination in large commercial poultry flocks.

Keywords: Fowlpox; Vaccination; Vaccination Failure; Wing Web Stab; Subcutaneous.

INTRODUCTION

Fowlpox (FP) Is an enzootic viral disease known for many years, and one of the earliest diseases studied due to the relative ease of isolation and identification of the virus (1, 2, 3). Fowlpox is widespread globally in domestic and wild birds (4, 5, 6), and characterized by two clinical forms: cutaneous and diphteric (7). Avian pox viruses (APVs) multiply in the epithelial cells cytoplasm, forming large typical cytoplasmic inclusion bodies (Bollinger bodies), causing hyperplasia, hypertrophy, degeneration and death of the infected cells (8, 9). If the multiplication takes place in the epidermis, the cutaneous manifestations are obtained, but when the pox virus replicates in the respiratory or the digestive tract epithelium, the diphteric pathological signs are observed. The cutaneous manifestations are considered relatively mild and characterized by multifocal proliferative skin nodules that appear mainly in non-feathered skin areas in the face and head (10). The cutaneous lesions are usually not fatal, by may cause severe stress caused by the disease itself, in the form of cutaneous pox may affect the eyes, beak and mouth decreasing the ability of the affected birds to eat and drink. In contrast, the diphteric manifestation is considered

severe and is characterized by proliferative necrotic lesions in the mucosal layer lining the respiratory and digestive systems, which lead to sever ulceration, hemorrhage, lumen obstruction, and secondary bacterial infections as the disease progresses (11).

Infection occurs by direct contact, swallowing or inhalation of contaminated dust or aerosol and by mechanical transmission (12, 13, 14). In commercial flocks the disease is characterized by a relatively slow rolling course, causing impaired performance (decreased growth and reduced egg production) and death. In severe cases the mortality ranges between 15-50% and is more severe in young birds. The incidence and severity of the disease varies between geographic areas depending on the climate (more common in tropical and subtropical climates), thus correlating with mechanical transmission by arthropods, housing, hygiene, and vaccination protocols (15).

Fowlpox Virus (FPV) belongs to the Poxviridea family and to the genus Avipoxvirus (16, 7), which currently includes 10 taxonomic species: canarypox, fowlpox, junco-pox, mynah-pox, pigeon pox, psittacine pox, quail pox, sparrow pox, starling pox and turkey poxviruses (17). FPV is a large, enveloped virus, which contains a double stranded DNA genome with a length of about 280kb (18) that encodes more than 250 genes (15). It is environmentally resistant compared to other enveloped viruses, possibly due to the presence of genes that protect the virus from environmental damage (19, 20). Even though it was one of the earliest studied avian viruses, much knowledge is still lacking regarding the phylogenetic relationships between Avipoxviruses (APVs), as well as regarding their host specificity. However, several phylogenetic studies show that the majority of APV's are host specific, and only a few can infect and cause disease in different species (21, 22, 23, 24).

In terms of immunity, there seems to be a good cross protection between some species (Fowlpox-Turkey pox-Pigeon pox), as a result of some conserved genes among APVs (15). It is interesting to note that integration of active Reticuloendotheliosis Virus (REV) pro-viral sequences into the FPV genome can be found in most field viruses, while in the vaccine strains there are only remnants of long terminal repeats (25, 26), the sequences of which are considered to be related to virulent characteristics of the virus.

APV vaccines were already developed by the end of 1920's (27). In fact, a vaccine against the disease was reported in the literature as early as 1928, using two APV species- FPV and pigeon pox (28), which today are related as antigenically similar species (29). Those vaccine strains, that were developed in that early period, are still the source of most of the vaccine strains commonly used today (over 70 commercial live attenuated vaccines). Therefore, knowledge about the exact origin of the strains, their attenuation process, and the relationships between them is extremely limited (29).

Development of recombinant vaccines based on FPV (rFPVs) began in the 1980's (30), and in the early 1990's rFPVs containing antigenic determinants of Newcastle Disease (ND) and Avian Influenza (AI) which were already registered in the USA (31, 32, 33).

Live attenuated vaccines against avian pox disease are widely used all over the world. According to the guidelines of the O.I.E. and commercial companies, Pox vaccines can be given by injection *in ovo* or to the chick after hatching (subcutaneous injection in the back of the neck) or by the wing web stab (WW) method on the farms. Testing for proper application of the vaccine given by the WW puncture method is based on the detection of a "Take" – (the appearance of a characteristic skin swelling or scab at the stab site about 5-10 days after the vaccination-OIE Terrestrial Manual (2018) (15). The duration of the protection induced by the vaccine probably includes a cellular and humoral response, is estimated to last 6-12 months after vaccination. Regarding other vaccination methods, mass vaccination in water or by aerosol administration was tested in several studies and was not shown to produce satisfactory results (34, 35).

Despite the extensive use of Pox vaccines, there are reports of outbreaks in commercial flocks vaccinated with the standard commercial vaccines (25, 26, 36, 37). In Israel, all light and heavy pullets are vaccinated during the rearing period once or twice against APV, using commercial attenuated vaccines given by WW stab application at different ages from 12 days to 15 weeks of age according to the vaccination programs used. Despite vaccination, in farms that maintain low biological safety (layers and turkeys flocks) or suffer from immunosuppression or stress (during molting), acute or chronic rolling outbreaks of Fowlpox occur (mainly in the dry form) (Fig 1, Fig 2), causing a serious welfare problem and damage to the flock's performance.

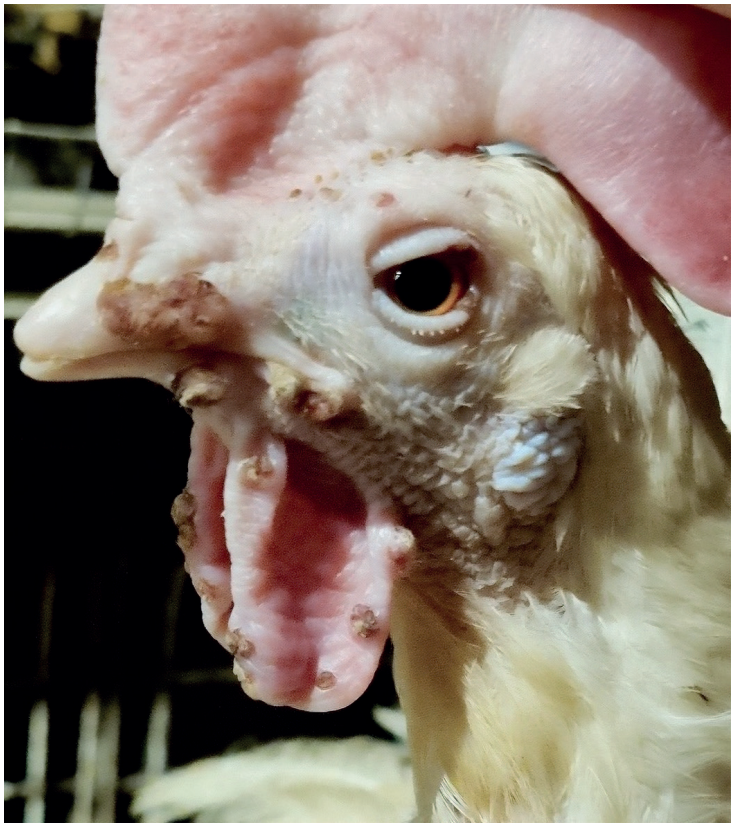

Figure 1. Cutaneous Pox lesions in layer hen vaccinated at 14 days old and 7 weeks, by the WW stab method

Figure 2. Cutaneous Pox lesions in a heavy breeder hen vaccinated at 5 weeks and 12 weeks by the WW stab method

Due to the lack of knowledge regarding phylogenetic relationships in the APV group, one of the hypotheses regarding the cause of the vaccine failure is heterology between the vaccine and field strains (25). According to this assumption, the heterology between vaccine and field strains leads to a low cross protection and therefore outbreaks in vaccinated flocks are possible. That heterology between strains may have been caused by the emergence of new FPV strains, by the presence of REV in the viral genome or by cross infection with different APV species due to the low specificity for the host that characterizes some of the strains (38, 39, 40, 22).

Despite the above mentioned, another possibility that must be considered as a cause of vaccine failure is the incorrect application of the vaccine in the field (41). Commercial fowl pox vaccines containing 1000-2000 doses per vial are usually diluted in 5 ml of the specific diluent. This means that the volume of the vaccine dose is only 0.005 ml to 0.0025ml. during vaccination, The vaccine is applied by WW puncture using a manual applicator with one or two needles or a semi-automatic Pox syringe (Fig 3).

The needles should have a calibrated grove (Fig 4. A, B) able to contain the desired amount of vaccine and deliver it into the pierced skin of the wing.

In large commercial flocks, the manual applicators are replaced in most cases by semi- automatic syringes that enable a much faster application. At least 90% of the pullets must be optimally vaccinated to provide adequate protection, and optimal vaccination using the WW stab method requires a highly skilled and trained vaccinating team and a good monitoring of the process.

In order to identify the main cause of the vaccination failure in some of the flocks in Israel, an epidemiological investigation of the outbreaks was carried out by the authors of this article. This investigation revealed that in the rearing pullet farms (four independent rearing farms) that provided some of the affected flocks, that the vaccination was carried out by different authorized and trained vaccinating teams, with vaccines from different commercial companies, from different vaccine batches, and that the vaccinated birds were of different ages at the vaccination time. The only factor that

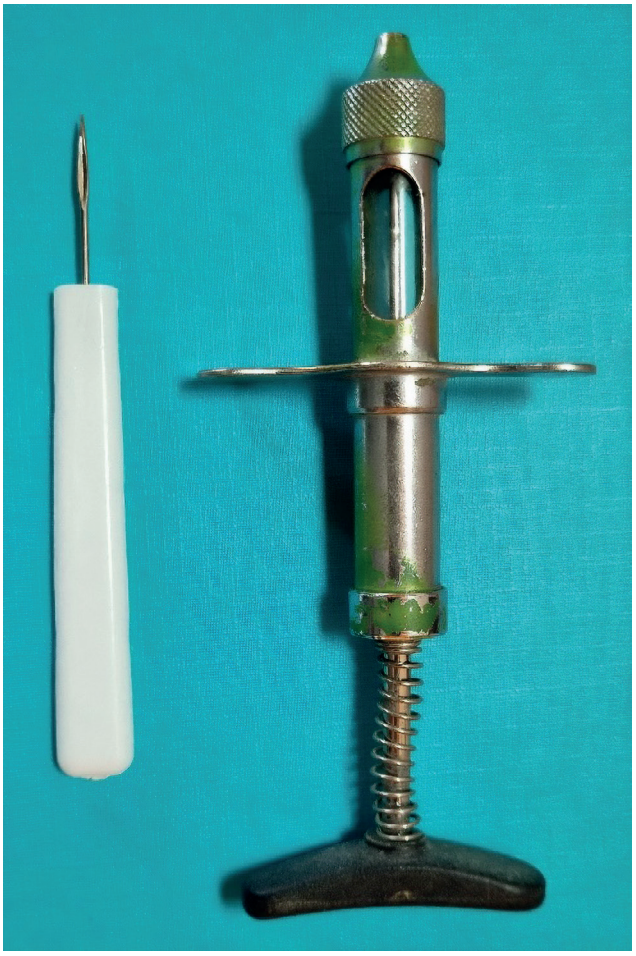

Figure 3. A manual applicator and a semi-automatic syringe for application of Pox vaccines by the Wing Web Stab vaccination.

was common to all the flocks was the method of application by the WW stab using semi-automatic Pox syringes.

In the next step, the (WW) application process carried out by different vaccination teams in the same pullet farms was closely monitored. A significant number of problems of application were identified during the vaccination of the flocks by this method and are further described in the discussion. All the vaccination teams used semi-automatic Pox syringes with one needle (in most cases) or two needles depending on the age of the birds. The authors found that in some cases the needles used had a very shallow grove and in other no groove at all, or that the groove was clogged with skin debris, leading to a very low volume of vaccine delivered to the puncture site. In many cases the semi-automatic syringes were held with the needles facing up and the diluted vaccine in the syringe container did not reach the grooves of the needles. Based on the above observations in all the monitored farms in this study there appeared to be a substantially inaccurate amount of vaccine delivered to the chickens and in some cases as much as 30%-40% of the vaccine remained unused (data not provided).

Examination of the "Take" after vaccination by WW stab, revealed that almost 100% of the birds were stabbed, but only 60-80% reacted locally with a clear "Take" at the stabbing point 4-7 days after vaccination (data not provided).

To attempt to overcome the problems commonly observed using the WW application for Pox vaccines, we tested and compared the safety and efficacy of Pox vaccination by subcutaneous injection to the WW stab method using commercial pox vaccines under controlled field conditions. These studies were carried out in layer pullets (Lohman and Dekalb lines) and heavy breeder pullet (Ross 308) rearing farms.

In this report we describe the results of those controlled field studies and the results of a long term (two years) follow up of the birds vaccinated by subcutaneous injection (SC) using different volumes of diluted Pox vaccine.

MATERIALS AND METHODS

Farms and Birds in the study:

The vaccination studies were carried out in commercial rearing farms and included replacement pullets for commercial eggs consisting of two chicken lines Lohman and Dekalb. The heavy breeder replacement pullets were Ross 308 and were reared in a heavy breeder rearing facility. All the Vaccinations were carried out by authorized and experienced vaccination teams.

All the revaccination "Challenge" tests and evaluation of the local reaction "Take" was carried out by the poultry veterinarians on the farms.

Equipment used for Pox vaccination:

Wing web (WW) stab vaccination: The WW stab vaccination in the commercial study flocks was carried out using semi-automatic syringes – ThaMa single needle Pox vaccinator (E. Nechmad, Petach Tikvah, Israel). The same type of syringe was used in all the rearing pullet farms from 14 days to 18 weeks of age. The "Challenge" by revaccination by Wing Web stab was carried out using a manual applicator or the same semi-automatic Pox syringe (Fig. 4).

Figure 4. Calibrated grooves in the manual applicator (A) and the semi-automatic Pox-Syringe (B).

Sub-cutaneous vaccination: The field studies were performed using three different types of syringes depending on the required volume of diluted vaccine to provide one dose/bird.

1. ThaMa 405 (E. Nechmad, Petach Tikvah, Israel) fixed dose automatic syringe with a 50 ml vaccine container (Fig. 5) was used for the subcutaneous injection of low volumes (0.05 or 0.1 ml/dose/bird.). The needles used were 20G ¼".
2. ThaMa 240 automatic syringes (E. Nechmad. Petach Tikvah, Israel) were used for the SC injection of standard volume (0.2-0.5 ml/dose/bird). The needles used were 20G/3/8".
3. Socorex 187 vial and tube feeding syringe (0.1-0.5 ml) (Socorex, Ecublens, Switzerland).

The diluent used for the wing web stab vaccination was the commercial diluent that was provided with the vaccine. To allow injection of higher volumes, the vaccine was initially diluted in the original diluent and then sterile saline was added to reach the required volume to provide an accurate dose per bird.

Safety and efficacy assessment:

To assess the safety of the novel application, adverse effects including development of Pox lesions, signs of disease and mortality were closely monitored in the study groups and were compared to the control groups from the day of vaccination for 4 weeks to enable the development of any adverse effect after vaccination.

To assess the efficacy of the SC vaccination under field conditions, a Pox challenge was simulated 14 to 21 days after vaccination (OIE- Fowlpox. Chapter 3.3.10) by puncturing the wing web with the commercial attenuated Fowlpox vaccine concentrated 5 times the dose per bird. Checking the "Challenged" birds 3-6 days post challenge for the appearance of a typical pox lesion at the site of puncture "Take". Development of a clear "Take' was an indication that the

bird did not develop any immunity post vaccination by SC injection.

Well protected birds should not develop any local reaction "Take" at the site of the stab wound. Chapter 3.3.10 (15).

Feasibility study-Safety

To test the feasibility of the novel application, a preliminary safety study was carried out (August 2020). The safety study took place in one flock of 14 days of age, replacement Lohman line layer pullets reared on litter. The group of chicks used to test the safety of the subcutaneous injection included 50 birds which were separated from the rest of the flock by fencing a small area in the chicken house (which contained enough food and water tools for the number of fenced pullets). The 50 separated pullets were vaccinated subcutaneously (SC) in the breast with one dose of standard commercial Pox vaccine Batch 1-051532 (Biovac – Or Akiva, Israel.) contained in 0.1 ml/bird using a manual 1ml syringe.

Preparation of the Pox vaccine for SC injection: The 1000 doses vial lyophilized Pox vaccine was diluted in 5 ml of the specific diluent provided. Sterile saline solution was added to the diluted vaccine to complete 50 ml of diluted vaccine to obtain 1 dose of 0.1 ml/bird.

The rest of the flock was vaccinated with the same vaccine using a ThaMa Fowlpox Syringe with one needle by the WW stab method. Six days after vaccination, the 50 pullets in this study were examined for the presence of a local reaction or swelling at the site of the SC injection. A close follow-up of the study group was carried out for a period of 4 weeks for detection of any adverse effects (local damage at the site of injection or development of Pox clinical signs or lesions) after the vaccination by SC injection.

In order to evaluate the protection obtained (efficacy) after the administration of the Pox vaccine by SC injection, all the 50 pullets in the study group were "challenged" 20 days post vaccination using a 5 times concentrated dose of the same Pox vaccine applied by WW stabbing method using a manual applicator with two needles and calibrated grooves.

Six days after the revaccination test (challenge with a high dose of vaccine by WW stabbing method) all the birds in this group were individually examined to detect the development of a local "Take" lesion to determine the protection provided by the subcutaneous vaccination.

Commercial Field Study- Number one:

The first commercial large-scale study took place in October 2020. Four thousand Dekalb line layer replacement pullets from the same parent flock and hatchery were included in this field study. All the pullets were housed in the same row of cages in a controlled environment chicken house.

At the age of 12 days 2000 pullets were vaccinated by subcutaneous (SC) injection in the breast, with one full dose of a commercial Pox vaccine – Batch 1-051532 (Biovac, Or Akiva, Israel.) diluted to obtain 1 dose (0.1 ml per chick) using an automatic low volume syringe, ThaMa 405 (E Nechmad, Petach Tikvah, Israel). The other 2000 pullets in the same row were used as the control group and were vaccinated with the same vaccine by the WW stab method using the single needle ThaMa Fowlpox Syringe (E Nechmad, Petach Tikvah, Israel). All the birds in the study were monitored daily for 14 days after vaccination to detect any adverse effects, signs of disease, Pox lesions or mortality. Fourteen days after vaccination, 100 birds from the SC vaccinated group and 50 birds from the WW vaccinated group were "Challenged" by revaccinatation by WW stab method using a 5 times dose of the same commercial Pox vaccine using a manual applicator with two calibrated needles. Five days later all the revaccinated birds were individually examined for the development of a local pox "Take" lesion.

Commercial Field Study – No 2:

The next field study under commercial conditions was carried out to test larger volumes of injection due to some technical problems observed using the low volume (0.05-0.1ml) syringes. To test the technical aspects of an increased volume for the subcutaneous injection, 8000 commercial Dekalb line layer pullets reared in cages in a controlled environment chicken house, were included in this study. All the 8000 pullets were from the same hatch day and originated from the same parent flock and hatchery. At the age of 7 weeks 4000 pullets were vaccinated by SC injection in the breast with one dose of a commercial Pox vaccine Batch 1-051533 (Biovac, Or Akiva, Israel) diluted in sterile saline solution to obtain 1 dose as 0.5 ml per chick using standard ThaMa 240 automatic syringes (E Nechmad, Petach Tikvah, Israel) and 20G/0.5" needles. The other 4000 pullets in the same battery were vaccinated

using the same vaccine diluted in sterile saline to obtain 1 dose of Pox vaccine as 0.1ml/chick using small volume syringes ThaMa 405 (E Nechmad, Petach Tikvah, Israel) with the same needle size.

Two weeks after vaccination, 50 birds vaccinated with the large volume /dose (0.5 ml/dose/chick) and 25 birds vaccinated with the low volume/dose (0.1ml/dose/chick) were "challenged" by revaccinating by the WW stab method applying a 5 times dose of the same commercial Pox vaccine using a ThaMa Fowlpox single needle syringe (E Nechmad,Petach Tikvah, Israel). All the 75 birds were examined 5 days later for the detection of a local "Take" reaction at the puncture site.

Commercial Field Study – No 3: (Heavy breeders replacement pullets):

After confirming the efficacy and safety of the Fowlpox vaccination using the novel application method by SC injection in the breast in replacement layer pullets, another field study in commercial heavy breeder pullets was carried out. The replacement breeders (Ross 308) were raised on litter in a farm consisting of four pullet rearing houses containing chickens of 13 weeks of age. The study group vaccinated by SC injection, consisted of two houses containing about 8400 birds each. All the pullets in these two houses were vaccinated by SC injection in the breast consisting of one vaccine dose of commercial Pox vaccine Batch 1-051533 (Biovac-Or Akiva, Israel) diluted in 0.2 ml of saline/dose/bird, using Socorex 187automatic syringes (Socorex, Ecublens. Switzerland) and 20G-3/8" needles.

The control group consisted of the other two houses at the farm containing 12500 birds (including 8500 females and 4000 males). All the birds in these houses were vaccinated by WW stab application with one dose of the same Pox vaccine using the single needle ThaMa Fowlpox Syringe. A few days after vaccination by the WW method a sample of the vaccinated birds were examined by the local poultry veterinarian for evaluation of the local reaction "Take". Fourteen days after vaccination, 20 pullets were randomly selected in each house of the study group (SC injection) and revaccinated by WW stab using a full dose of the same Pox vaccine and examined three days later by the veterinarian for the presence and evaluation of a "Take" in the stabbed wing.

RESULTS

Feasibility preliminary study (Safety):

None of the pullets in the experimental group showed any adverse effect or evidence of disease, or any other lesions characteristic of Fowlpox disease during a period of four weeks.

The "challenge" test carried out by revaccination by the WW stab method using a 5 times dose of the Fowlpox vaccine demonstrated that none of the 50 vaccinated birds by the SC injection method developed any local reaction "Take" after the WW revaccination challenge, indicating a good immune response and protection after the SC vaccination.

Commercial Field Study – No 1:

As described in the materials and methods section, this study focused on comparing the safety and efficacy of SC injection compared the common WW stab method both applied by two experienced and qualified vaccination technicians in 4000 pullets under commercial conditions. In this study, no adverse effects (mortality, Pox Lesions, lesions at the site of injection in the breast, etc.) was observed in any of the SC or the WW vaccinated pullets. Furthermore, both groups showed very similar efficacy results after the "challenge" by revaccination with a 5 times dose of Fowlpox vaccine. The results were 96% (84/87) in pullets from the SC vaccinated group and 95% (42/45) in pullets from the control group vaccinated by the WW stab which did not show any sign of a "Take" indicating a good application and protection in both groups.

Two important and practical points were observed in this study:

Firstly, the vaccination of the 2000 chicks by SC injection was much faster (2000 chicks in one hour) compared to the WW application (2000 chicks in 1 hour and 30 min) even though both applications were performed by two experienced vaccination technicians working at the farm. Secondly, during the vaccination with the low volume syringe (0.05-0.1 ml) it was difficult to assess if the vaccine was flowing properly and as a result, the vaccinator had to check several times during the vaccination if the syringe was providing the right volume of vaccine.

Commercial Field Study – No 2:

This field commercial study was focused on comparing the technical and practical aspects and efficacy of the application

Table 1: Summary of three studies carried out under commercial conditions to test the safety and efficacy of Pox vaccination by Subcutaneous injection using different volumes of injection/dose.

	Commercial study 1		Commercial Study 2		Commercial Study 3	
	WW	**SC 0.1ml**	**SC 0.1ml**	**SC 0.5ml**	**WW**	**SC 0.2ml**
Number of Chickens	2,000	2,000	4,000	4,000	12,500	16,700
Adverse effects, Pox lesions or mortality	Non	Non	Non	Non	Non	Non
Revaccination "Challenge" using a X5 dose Pox Vaccine by WW Stab in WW or SC vaccinated birds						
	Commercial study 1		**Commercial Study 2**		**Commercial Study 3**	
	WW	**SC 0.1ml**	**SC 0.1ml**	**SC 0.5ml**	**WW**	**SC 0.2ml**
Protected "No Take"	42/44 95%	84/87 96%	21/25 84%	49/50 98%	ND	40/40 100%
Mild local inflammation at puncture site	2/44	3/87	4/25	1/50	ND	0/40
Not Protected Well developed "Take"	0/44	0/87	0/25	0/50	ND	0/40

ND-Not Done

of a Pox vaccine by SC injection in the breast. The birds were vaccinated by the same vaccination team using different syringes providing different volumes of injection per dose: ThaMa 405 (E. Nechmad, Petach Tikvah, Israel) – using 0.1ml/dose or ThaMa 240 (E. Nechmad, Petach Tikvah, Israel) – using 0.5ml/dose.

The results in this study indicated that if the low volume of 0.1 ml/dose/bird by SC injection was applied properly, there was no difference in the efficacy when compared to SC injection using a larger volume of 0.5ml/dose/bird. In accordance, both groups vaccinated by the SC injection showed a very good protection as 100% in both groups did not show any "Take" lesion after the challenge by revaccination with a high dose of Pox vaccine by WW stab.

Commercial Field Study – No 3:

This field commercial study was focused on assessing the efficacy of SC application (compared to the WW stab technique) in heavy breeders (Ross 308) replacing pullets. All the birds on the farm were vaccinated with the same Pox vaccine by the same experienced vaccination team and the monitoring was carried out by the veterinarian in charge.

Examination of the control birds vaccinated by WW puncture by the local vaccination team revealed that only 78% (31/40) of the vaccinated pullets showed a local reaction of a "Take" after vaccination by the traditional WW stab method. In the birds vaccinated by SC injection by the same team, and "challenged" by revaccinating 14 days later by WW stab method, no sign of a "Take" were identified in any of the revaccinated birds (40/40) indicating that the vaccination by SC injection induced a uniform and complete protection in 100% of the pullets. The results of all the commercial field studies are summarized in Table 1.

DISCUSSION:

Sporadic cases of Fowlpox are observed in vaccinated flocks all over the world including Israel. In most of these cases the outbreaks are relatively mild affecting 10%-30% of the flock. The Pox lesions are usually cutaneous and localized to the face, eyelids, wattles, and comb with no diphteric lesions. In most of these cases mortality is very low, however there is a clear welfare issue and performance is negatively affected.

Flocks suffering from Pox outbreaks during production or molting, should be revaccinated with Pox vaccine to stop the rolling of the disease within the flock causing severe stress and economic losses.

The epidemiological investigation carried out in several rearing pullet farms in Israel, to define the reason of the vaccine failures (Fowlpox outbreaks during production or molting) observed in Fowlpox vaccinated flocks, revealed that the vaccination by wing web stabbing suffered from many technical problems leading to low uniformity of the development of immunization and protection of the birds.

In large commercial rearing farms, the Fowlpox vaccine is usually applied by the WW stab technique using a one

or two needle semi-automatic Pox syringe. In some cases the needles used have a very shallow grove or no groove at all. Even in the case where the needles were examined and approved before use, we found after vaccination of several hundred birds, that the grooves of the needles were clogged with skin debris, leading to a very low volume of vaccine delivered to the puncture site.

In other cases, we observed that the semi-automatic Pox syringes were held with the needles facing upwards and the diluted vaccine in the syringe container did not reach the grooves of the needles. Independently from the vaccination team, in all the monitored farms in this study it appeared that the WW application delivered an inaccurate amount of vaccine to the chickens and in some cases as much as 30%-40% of the vaccine remained unused.

Examination of the "Take" after vaccination of the pullets flocks by experienced and authorized teams in different farms using the WW stab method, revealed that in most cases almost 100% of the birds were stabbed in the wing. However, we found that only 60-90% of the vaccinated birds reacted locally with a clear "Take" at the stabbing point four to seven days after vaccination. The results obtained after vaccination by SC injection of several thousands of birds in commercial flocks with Fowlpox vaccines diluted in sterile saline solution (one full dose/bird), strongly supported the assumption that this vaccination method was safe as no adverse effects of any kind were observed in any bird within the vaccinated flocks.

Regarding application and efficacy, we found that the subcutaneous injection (SC) in the breast using automatic syringes was faster and more reliable than the WW stab method, enabling therfore the use of 100% of the vaccine doses (one dose/bird) in all the flocks in the study compared to the WW stab application.

We found that the volume of injection between 0.1-0.5 ml/dose/bird if applied properly using different types of syringes, had no effect on the efficacy if the birds received the required one full dose of the vaccine. Technically we observed that vaccination using syringes with very low volumes (0.05-0.1ml), required the vaccinator to check continuously if the syringes were delivering the vaccine. On the other hand, the SC injection using syringes with larger volumes from 0.2ml to 0.5ml provided a better control and accuracy of the administration of the vaccine dose.

The "challenge" of the birds vaccinated by SC injection using a five times dose of Fowlpox vaccine administered by wing web stab (WW) demonstrated a very uniform and efficient immunization of the flocks vaccinated by SC injection.

During the last two years there has been a change that is gradually spreading in the poultry industry in Israel, in which farms and private vaccination teams apply the commercial attenuated pox vaccines using the subcutaneous injection instead of the wing web stab method. It is estimated that to date, more than 3 million birds (layers and breeders) have been vaccinated with Pox vaccines by SC injection with not a single report of adverse effects during the rearing and production stages. Up to now, no reports of outbreaks of Poxvirus have been reported in Israel in any of the vaccinated flocks using the SC injection application.

The conclusion of this field studies strongly supports that the application of Fowlpox vaccines by subcutaneous injection is a safe, more practical and reliable alternative than the wing web stab (WW) application in large commercial flocks.

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Re-emergence of Bovine Ephemeral Fever in Turkey in 2020 after an 8-Year Absence: A Molecular Analysis Study

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ABSTRACT

Bovine ephemeral fever (BEF) is a vector-borne disease of ruminants in tropical and subtropical areas, leading to significant economic losses to the cattle and milk industry in many countries, including Turkey. Cattle showing high fever, stagnation, and recumbency have been extensively reported in Turkey's south and south eastern regions in 2020. Here, the genetic analysis and molecular epidemiology of the virus obtained from the last BEF outbreak were investigated. Of 32 symptomatic cattle selected from three provinces in Turkey, 23 were positive for bovine ephemeral fever virus (BEFV), and three entire G genes were identified by sequencing. The new BEFV isolates were genetically similar to some Turkish and Israeli isolates from 2008 and 2012 (>98 nucleotide (nt), 97% amino acid (aa)), and phylogenetic analysis based on the surface glycoprotein (G) gene revealed that BEFV isolates are of Middle Eastern origin. The G protein amino acid alignment showed that BEFV circulated in the same region with minor differences over the years. In this context, we recommend closely monitoring BEF outbreaks in neighbouring countries and prompt vaccination of susceptible cattle in areas at risk for BEFV in Turkey in the event of an outbreak.

Kew words: BEF; Genetic Characterization; Epidemic; Report; Turkey.

INTRODUCTION

Bovine ephemeral fever, also known as BEF, 3-day sickness, 3-day fever, bovine enzootic, bovine influenza, stiffseitke, or dragon boat) is an acute febrile illness of cattle, water buffaloes, and occasionally other ruminants, transmitted by arthropod vectors such as mosquitoes and biting midges (*Culicoides* spp.) (1). Bovine ephemeral fever, is widespread in tropical and subtropical areas of Africa, Asia, Australia, and the Middle East, causing an acute systemic inflammatory response characterized by biphasic fever, lymph node enlargement, salivation, nasal and ocular discharges, subcutaneous edema, tachycardia, respiratory distress, muscle stiffness and tremors, lameness and paralysis (2). In the epidemic season, infection and morbidity rates are typically high (nearly 100%). Nevertheless, mortality rates generally remain low (<2%), except for a few reports in Turkey and China, where they reached 10–20% (3, 4). The economic burden of BEF may be considerable and are due primarily to a sudden drop in milk production in dairy cattle, loss of condition and infertility in beef cattle and the immobilization of water buffalo used for draught power (5).

Bovine ephemeral fever virus (BEFV), the etiological agent of BEF, belongs to the family Rhabdoviridae, genus Ephemerovirus, and species Bovine ephemeral fever virus (5). It has a single-stranded, negative-sense RNA genome of 14,900 nucleotides (nt) in size that consists of 10 ORFs, which are 3'-N-P-M-G-[GNS-α1-α2-β-γ]-L-5' (6). The BEFV virion, which typically displays rhabdovirus

bullet-shaped morphology, contains five structural proteins: Nucleoprotein (52 kDa), tightly associated with the viral genome and most abundant in the virion; phosphoprotein (43 kDa), essential for transcription and replication. Matrix protein (29 kDa), which plays a critical role in virus maturation and budding; glycoprotein (81 kDa), which is a virion membrane surface protein and contains the major neutralizing antigen; and L protein (180 kDa), which is a multifunctional enzyme (1, 5). Apart from the structural proteins, the viral genome encodes five non-structural proteins, except for α1, which acts as a viroporin, and α3, which plays a role in apoptosis. The functions of other proteins (GNS, α2, and β) have not yet been elucidated (7, 8).

The BEFV's glycoprotein (G) gene has been sequenced most frequently. A phylogenetic analysis of this gene showed that the isolates fall into four groups based on where they were isolated: Africa, Asia, Australia, and the Middle East (9). BEF has been documented in Iran, Israel, Jordan, Syria, and Iraq, as well as Saudi Arabia and Turkey, and has been described in Egypt and Palestine (10).

The first reported BEF outbreak in Turkey was documented in 1985 in Anatolia's central, southern, and south-eastern regions, followed by outbreaks in 1999, 2003, 2008, and 2012 (4, 11). Although most outbreaks occurred in the southern part of Anatolia, the seroprevalence of BEF was reported as 2.5-15.3% and 13.5% in the western provinces of European Turkey and the central Black Sea region, respectively (12, 13). BEF outbreaks in Turkey occurred periodically every 4-5 years from 1999 to 2012, (4, 11) and each time they had the potential to spread rapidly in the south and southeast of Turkey, threatening cattle life and causing significant yield and economic losses. Therefore, BEF may be considered one of the important seasonal disease in the southern and south-eastern coastal regions of Turkey. While it seems more or less predictable when BEF will emerge, the situation in the last outbreak was unexpected. Eight years later, in 2020, the previous BEF outbreak in Turkey was markedly reported by veterinarians in Turkey's southern, south-eastern, and eastern provinces in late summer and early autumn, when temperatures exceeded 40°C and mosquito populations increased.

This study investigated the genetics and molecular epidemiology of the virus that was isolated in Turkey during the BEF epidemic of 2020.

MATERIALS AND METHODS

Sample collection and preparation, ethical statement

From the beginning of September to mid-December 2020, a total of 32 tubes of heparinized blood were collected from the jugular vein of symptomatic (high fever, sternal recumbency, stiffness, and oral and nasal discharge) cattle populations in Kahramanmaraş, Hatay, and Elazığ provinces located in the southern and eastern Anatolian region of Turkey. The blood samples were transferred in a constant cold chain to the Virology department of the Faculty of Veterinary Medicine of Firat University. Heparinized blood was centrifuged at 4500 rpm at 4°C for 15 minutes using a Hettich 32R centrifuge (Hettich, Tuttlingen, Germany) to separate the plasma. The obtained plasma was stored at -80°C until virus isolation and viral genome detection were carried out.

The Animal Experiments Ethics Committee of Firat University, gave permission No. 423048, for all procedures that were performed on the live animals.

Viral genome detection and virus isolation

Viral RNA isolation from plasma samples was performed with the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. One-step RT-PCR was applied to detect the positivity of clinical samples and performed as previously described with specific partial G gene primers (14). BEFV was separated from the processed blood cells in the same way as descibed by Abayli *et al.* (15). Briefly, African green monkey kidney epithelial cells (Vero E6, obtained from the American Type Culture Collection (ATCC, CRL 1586) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, MO, USA) supplemented with 15 mM/L HEPES (Thermo Fisher, MA, USA), 1.5 gr/L sodium bicarbonate (Merck, Darmstadt, DE) L-glutamine (Sigma-Aldrich, MO, USA), 100 U/mL penicillin (Sigma-Aldrich, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich, MO, USA), and 10% fetal bovine serum (Gibco, MA, USA) at 37°C in 5% CO2. After removal of the medium, 70% confluent Vero E6 cells were washed twice with PBS, inoculated with processed blood cells, and incubated for one hour at 37°C. At the end of the incubation, the inoculum was replaced with DMEM supplemented with 1% FBS (Gibco, MA, USA). Vero E6 cells were blind passaged six times and examined under the microscope daily for cytopathic effects.

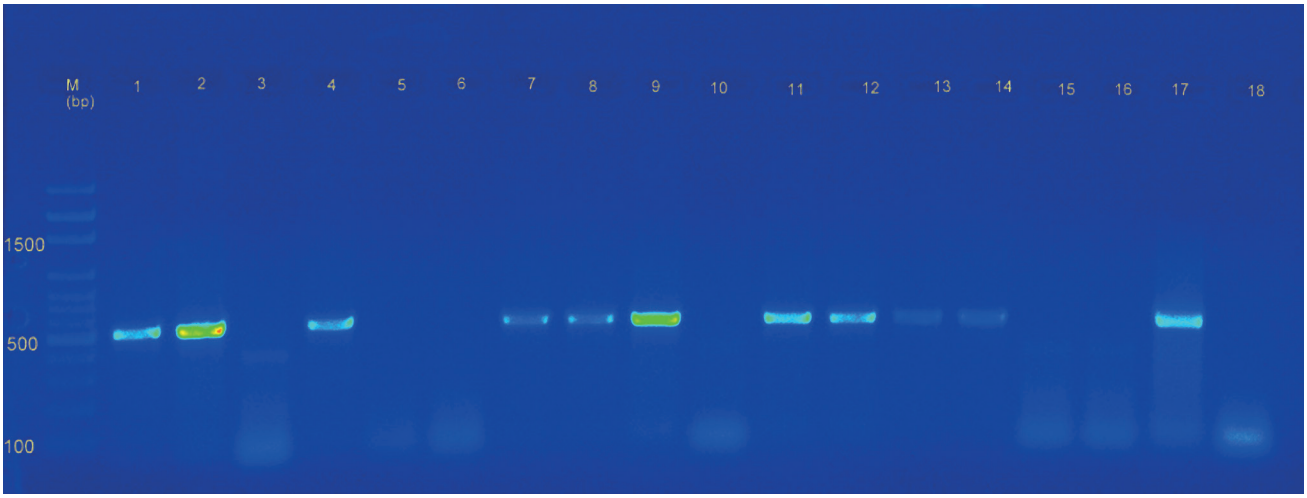

Figure 1. Agarose gel (1.5% w/v) electrophoresis after PCR screening from field samples. **M:** Solis Biodyne 100 bp DNA ladder.

RT-PCR for the full-length G gene, sequencing, and phylogeny

RNA from viruses were used to make a one-step RT-PCR kit that amplified the full-length G gene (Thermo Fisher, MA, USA). Briefly, the assay was carried out in a 50 μL reaction mixture containing five microliters of viral RNA (50 ηg/uL), three microliters of each primer (20 ρmol), ten microliters of the 5× buffer, two microliters of 10 mM dNTPs, two microliters of a mixture of SuperScript III reverse transcriptase and platinum Taq DNA polymerase and ultrapure water (all the reagents were provided in the kit) (15). After the reverse transcription step at 50°C for 30 min, amplifications were carried out at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min. The amplification products were analyzed in 1.5% agarose gel electrophoresis with ethidium bromide (Sigma-Aldrich, MO, USA) using a 100 bp DNA ladder (NEB, MA, USA). Electrophoresis was performed in an agarose gel electrophoresis system (Thermo Fisher, MA, USA) for 40 min at 120 volts, and the PCR products were visualized under UV light. The PCR fragments were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced in both orientations with the complete G gene primers. This stage was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems, CA, USA) using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). The resulting bidirectional nt sequences were aligned, edited, verified with BLASTN, and submitted to the GenBank database (OQ134925-7).

Thereafter, nucleotide and amino acid sequences (aa) were aligned and compared with strains selected from GenBank using Clustal W software. Sequence data which had been submitted to the GenBank Nucleotide Sequence Database. Phylogenetic trees were generated using Molecular Evolutionary Genetics Analysis software version X (MEGA X), and the Maximum Likelihood method with 1000 bootstrap replicates (16).

RESULTS

Viral genome confirmation and virus isolation

After RT-PCR, of 32 heparinized blood samples, 23 (71.8%) had the predictable DNA fragment size (520 bp). After agarose gel electrophoresis, some of the positive samples are shown in Fig 1. Of 23 PCR-positive samples, 12 were confirmed by sequencing. Sequencing results were categorized into three. G genes randomized three samples from different categories were amplified by RT-PCR and deposited in GenBank by editing [TR-Hatay-2020-BEFV (OQ134925), TR-Maras-2020-1-BEFV (OQ134926), TR-Maras-2020-2-BEFV (OQ134927)].

No viral RNA and CPE could be detected at the end of the 6th blind passage in Vero E6 cells.

BEFV G gene sequencing

Based on G gene sequence analysis, three new BEFVs were identified (TR-Hatay-2020-BEFV, TR-Maras-2020-1-BEFV, and TR-Maras-2020-2-BEFV) (99.5-99.9% nt and 99.0-99.8% aa). The new BEFVs were genetically very similar to the Turkish isolate, the BEFV/Ad12/TUR (99.4-99.9%

Table 1: The nucleotide identity of existing new BEFV strains with some Israeli, Iranian, Egyptian, and Indian strains

	BEFV stains/isolates (Accession numbers)	TR-Hatay-2020-BEFV	TR-Maras-2020-1-BEFV	TR-Maras-2020-2-BEFV
Israeli	2008-Israel (JN646090)	99.0	98.8	98.4
	ISR00_2000 (JN833630)	97.0	96.9	96.5
	ISR01_2001 (JN833631)	97.1	97.0	96.7
	ISR04_2004 (JN833632)	97.0	96.9	96.6
	BEFV/Israel/2006 (MN078236)	97.3	97.2	96.9
	ISR10/1_2010 (JN833633.1)	96.5	96.4	96.0
	ISR10/2_2010 (JN833634)	96.4	96.2	95.9
Iranian	Khuzestan-2018 (MZ511169)	97.4	97.3	97.0
	2018-Ahvaz (MT274593)	96.2	96.2	96.2
Egyptian	EGY12 (KJ729108)	95.1	95.0	94.8
	Damietta2/Egy/2017 (MH939251); Damietta5/Egy/2017 (MH939254.1); Dakahlia3/Egy/2017 (MF968902); Dakahlia1/Egy/2017 (MF968900); Kafr_El-sheikh2/Egy/2017 (MF968904.1)	96.0	95.9	95.5
Indian	IND/JBL/BEV/2018 (MH933863.1); IND/BH/BEV/2018 (MH933862.1)	97.2	97.2	97.2
	IND/IDR/BEV/2018 (MH933864)	96.9	96.9	96.9
	IND/INDR/BEV/2019 (MN688612.1)	95.7	95.56	95.1

nt and 97.9-99.9% aa), followed by the other Turkish isolates (2008/TR/CP77, 2008/TR/CP62, and 2008/TR/CP60) with high identity ratio (98.7-99.2% nt and 97.5-99.2% aa). The new BEFVs had lower (96.9-98.9% nt and 96.8-98.8% aa) genetic relatedness to the two BEFVs reported by different investigators in the 2020 epidemic (TR/NO3/URF/2020 and TR/D2/URF/2020) (17).

The nucleotide identity of existing new BEFV strains, when compared with BEFVs from different geographic regions, shared a high identity with some Israeli, Iranian, Egyptian, and Indian strains (>95.0% nt; >97.0% aa) (Table 1). According to the same analysis results, this rate was 89.0-90.5% for the Australian reference strain named BB7721 (AF234533) and other Australian strains (MN026882-3; MN026898-9; MN026888); 85.3%-90.2% for Saudi Arabian strains (LC017738); 86.0-87.0% for South African strains (MN026880-1; MN026884; MN026887; MN026890-1; MN026896; MW463337; MW512963); 91.5-92.4% for Iraqi strains (MW600731-3).

Amino acid substitutions

The glycoprotein amino acid sequence of the new BEFV strains had substitutions at some positions compared to the Australian reference strain (BB7221): 16 (Leu-Phe), 18 (Lys-Glu), 72 (Ala-Asp), 83 (Arg-Lys), 198 (Glu-Lys), 200 (Ile-Val), 216 (Asn-Ser), 224 (Lys-Thr), (Lys-Glu), 223 (Asp-Glu), 237 (His-Arg), 249 (Lys-Arg), 250 (Asn-Ser), 305 (His-Pro), 311 (Thr-Pro), 333 (Arg-Ser) 360 (Arg-Lys), 366 (Asn-Ser), 399 (Val-Ile), 410 (Gln-Leu), 419 (Gly-Arg), 426 (Thr-Ser), 435 (Asn-Thr), 436 (Arg-Lys), 459 (Leu-Ile), 465 (Asp-Glu), 480 (Val-Ile), 486 (Arg-Lys), 499 (Asn-Ser), 503 (Lys-Thr), 567 (Ser-Asn), 570 (Arg-Ser), 580 (Thr-Ile), 581 (Thr-Ala), 583 (Glu-Gly) and 586 (Arg-Lys).

The amino acid sequence of new BEFVs was compared with those of Turkish isolates and determined substitutions. Lys18Glu was seen only in three new BEFVs and BEFV/Ad12/TUR. Some amino acid substitutions were unique for TR-Maras-2020-2 (Asp223Glu, Lys224Pro, Thr311Pro, Arg333Ser, and Arg360Lys) and for TR-Maras-2020-1 and TR-Maras-2020-2 (His305Pro). Some amino acid substitutions (Ile98Thr and Thr503Ala) in isolate BEFV/Ad12/TUR could not be detected in other Turkish strains/isolates. Ala429Thr was found in both TR/NO3/URF/2020 and TR/D2/URF/2020 isolates obtained in 2020, whereas substitution Ala427Glu was detected only in TR/D2/URF/2020. The G protein amino acid alignment with the

new Turkish BEFV strains and some others is shown in Fig 2.

Phylogenetic analysis

With the G gene nt data of 161 BEFV strains/isolates from the Middle East, East Asia, South Africa, and Australia/Philippines, a phylogenetic tree was prepared (Figs. 3 and 4). The four phylogroups/lineages were grouped as the Middle East, East Asia, South Africa, and Australia/Philippines. Turkish strains and isolates were found to be divided into two lineages (Middle East and East Asia). The Middle East lineage was found in all Turkish strains and isolates between 2008 and 2020. Some strains/isolates from 2012 were included in the East Asian lineage, exhibiting distant genetic characteristics from other Turkish BEFVs. According to the results, Turkish strains/isolates clustered in the East Asian lineage were 2012/TR/CU16, 2012/TR/CU15, 2012/TR/CP3, Mersin/Silifke6251-1/Turkey2012, TR/ADA-2/2012, TR-Etlik-2-BEF-2012, TR/ADA-1/2012, Adana5918-15/Turkey/2012, and Mersin/Silifke625-6/Turkey2012. BEFVs obtained in 2020 were in the same branch (Middle East lineage) but were in different sub-clusters. New Turkish BEFVs were sub-clustered along with BEFV/Ad12/TUR, 2012/TR/ADYMN, 2012/TR/Skr.1, 2008/TR/CP77, 2008/TRCP62, 2008/TR/CP60 2008-Israel, and IND/INDR/BEV/2019.

DISCUSSION

Bovine ephemeral fever occurs seasonally in a significant area of the world, including most of Africa, the Middle East, Asia, and Australia, posing a serious economic burden to the livestock industry in many countries, including Turkey (1, 4, 9, 11). The glycoprotein (G) of BEFV has four independent antigenic regions (G1, G2, G3, and G4) that cause the host to produce neutralizing antibodies (18-20). This has led to its extensive study in phylogenetic studies, making it a representative of the whole genome (1, 15, 21). In this study, the G gene of viruses from the last BEF epidemic in Turkey in 2020 was sequenced and analysed. When the G gene nucleotide and amino acid sequences were examined, the new BEFV strains showed high similarity to the strains of Egypt, Israel, Iran, and India (>95.00% nt, >97.00% aa). These results were also supported by the phylogenetic analysis, which revealed that BEFVs included four phylogroups/lineages (Middle East, East Asia, South Africa, and Australia/Philippines). As previously, most of the viruses from the countries above were included in the Middle Eastern lineage in the phylogenetic tree (4, 15). These results confirm a significant correlation between geographical distance and phylogenetic relationships (4, 22-24). The three new BEFVs shared a high genetic close relatedness (>96% nt) with the Israeli and Iranian strains. It is interesting that Indian strains also exhibited a high genetic relatedness to them, which is a significant finding in terms of the epidemiology of the virus. These results support the scenario of the inter-continental BEFV virus spread (22, 24). Wind transport of infected vectors over long distances is a scenario considered for past BEF epizootics (24-26). In the worst-case scenario, an outbreak in Africa or the Middle East could spread to Turkey via Egypt, Israel, and the Syrian corridor. Similarly, the virus could be transmitted to India and China via Iran and complete its cycle in the Africa-Middle East-East Asia triangle. In this context, vector control should be one of the measures taken to prevent the spread of BEFV. BEFV can be spread through uncontrolled animal movements or the animal trade (1, 24). In the 2012 epidemic, BEFV strains from two different origins circulated, in Turkey, suggesting the recent animal trade from China to the Middle East (1). Increasing virus controls with BEFV screening in the host and limiting free animal crossings between Turkey and border countries may limit the spread of BEFV.

Vaccination is recognized as the most effective measure to prevent BEF and is used with attenuated live vaccines for this purpose in Turkey (1, 27). It would be appropriate to follow up on BEF outbreaks in neighboring countries and to vaccinate susceptible cattle in regions that pose a risk for BEFV in Turkey at the time of the BEF outbreak. Similarly, it is essential to follow up on the antigenic characteristics of the viruses in the epidemic. Here, epitopic regions of BEFVs from the last BEF epidemics were also analyzed. G protein aa alignment of the three new BEFVs with 12 selected BEFVs showed that epitopes other than G3b were mainly conserved. Turkish BEFVs from the last epidemic had substitutions at positions 223 (Asp to Gly) and 224 (Lys to Thr; Lys to Pro) of the G protein corresponding to the G3b epitope. It has been demonstated that there is a single serotype of BEFV present worldwide (1, 2), however heterologous BEFV isolates obtained from different geographic areas or at other times exhibit different cross-neutralization (28, 29). Although BEFV is the only serotype, minor changes can be seen in epitopic regions (11, 30, 31). For instance, in Australia, four

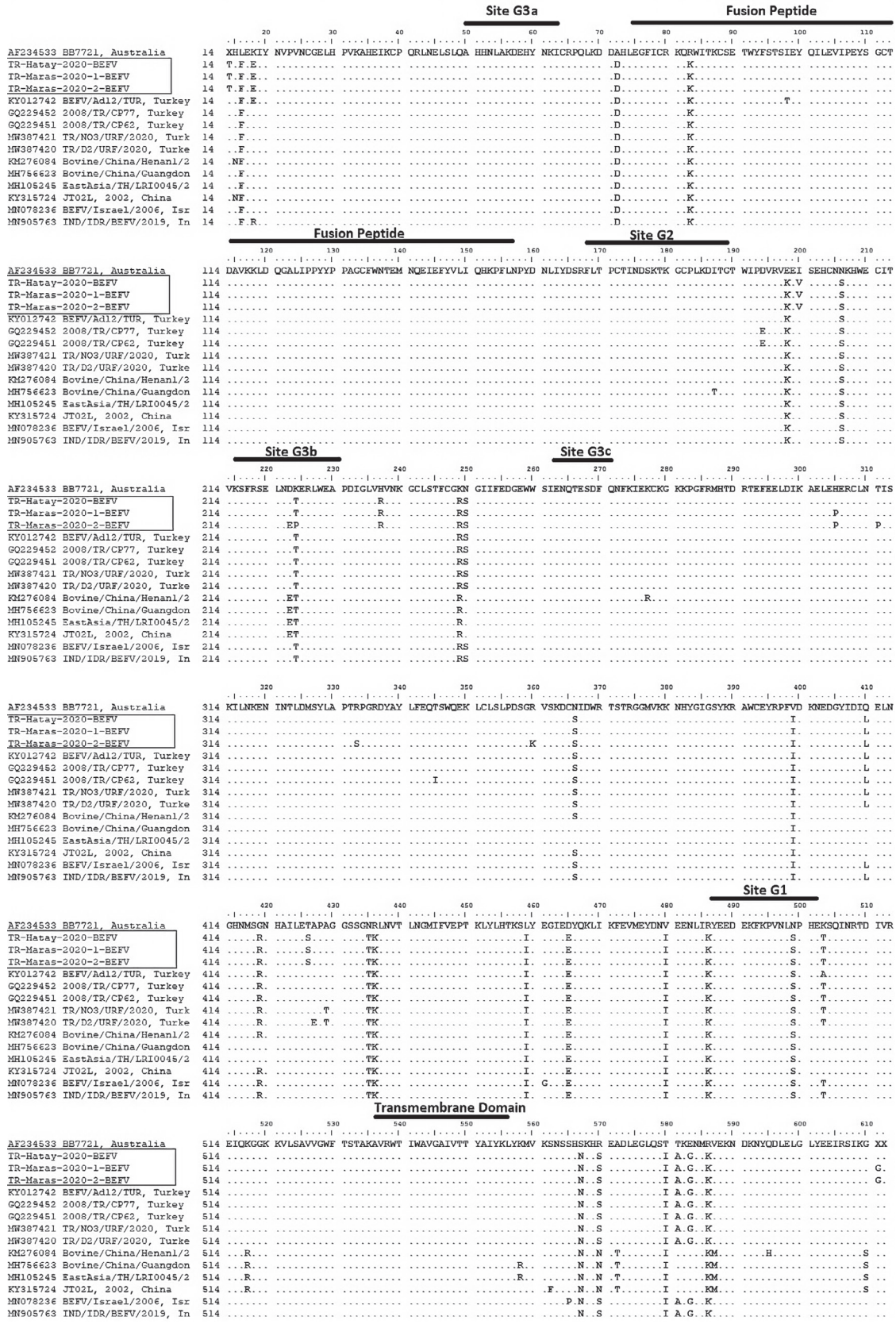

Figure 2. The G protein amino acid alignment with the new Turkish BEFV strains and some others

Figure 3. Phylogenetic tree constructed with G gene nucleotide data of 161 BEFV strains/isolates selected from Genbank.

Phylogenetic tree was created with Mega X software in 1000 replicates using Maximum Likelihood method and Tamura-Nei model. Filled circles indicate BEFV strains obtained from this study, and unfilled circles indicate other Turkish strains/isolates

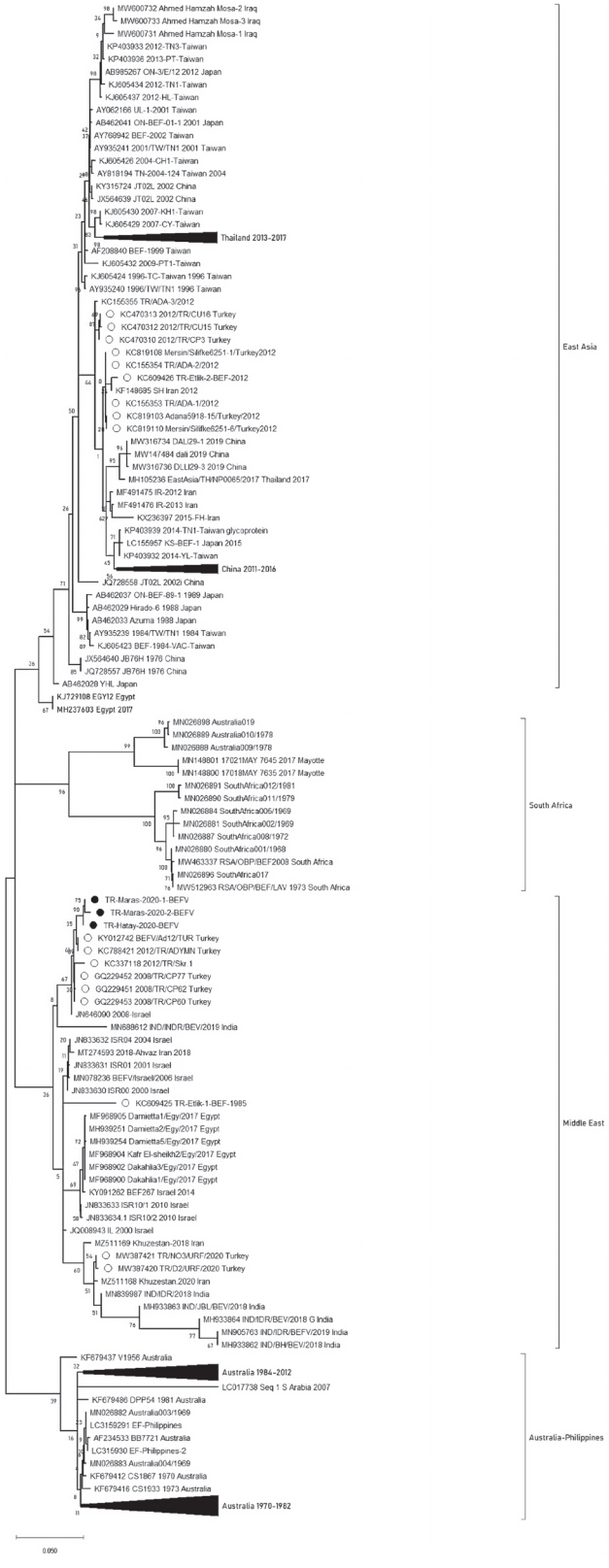

Figure 4. Phylogenetic tree constructed with G gene nucleotide data of 161 BEFV strains/isolates from similar or different geographies. Phylogenetic tree was created with Mega X software in 1000 replicates using Maximum Likelihood method and Tamura-Nei model. Filled circles indicate BEFV strains obtained from this study, and unfilled circles indicate other Turkish strains/isolates.

subtypes have been identified based on variations in the epitopes G3a and G3b (31). Whether substitutions in the G3b region of new BEFVs dominate or cause phenotypic changes should be further investigated, and existing vaccine strains should be updated in case of significant differences.

In conclusion, after eight years, the BEF epidemic again broke out in Turkey, showing that the risk of BEFV continues to be present in Turkey. The fact that the G protein of the BEFVs studied here was frequently the same and that only the G3b epitope was different shows that the virus is still circulating with only minor changes in geography.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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First Report of Concurrent Infection of Canine Kobuvirus and Canine Distemper Virus in a Diarrheic Dog in India

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ABSTRACT

Canine Kobuvirus (CKoV) has been recently reported in many countries such as United Kingdom, China, Thailand, United States of America and Italy. In the present study, dogs suffering from gastroenteritis were screened for the presence of Canine Distemper Virus (CDV) in their fecal samples using RT-PCR. The dogs found to be positive for the presence of CDV in the diarrheic fecal samples were subjected to next generation sequencing (NGS) for the whole genome analysis. From one sample the whole genome of Canine Kobuvirus (CKoV) along with partial genome sequences of CDV was obtained. Phylogenetic analysis based on the complete nucleotide genome sequence of CKoV, revealed that the virus had 91-95% nucleotide identity with the Chinese, Japanese and UK strains. Similarly, the phylogenetic analysis based on the partial genome sequence of the CDV isolate showed 97% nucleotide identity with other Indian isolates and Chinese strains. To the best knowledge of the authors, this is the first report of detection and molecular characterization of CKoV in a domestic dog in India. Our result highlights the concerns to veterinarians that diarrhea in dogs may also be due to Canine Kobuvirus infection in addition to the other potential pathogens, and should not be ignored.

Keywords: Canine Kobuvirus; Canine Distemper Virus; Next Generation Sequencing (NGS); Whole Genome.

INTRODUCTION

The *Kobuvirus* which has been identified as a new genus in the family *Picornaviridae*, consists of three species, Aichivirus A (formerly Aichivirus) (1), Aichivirus B (formerly bovine kobuvirus) (2) and Aichivirus C (porcine kobuvirus) (3). The species Aichivirus A consists of four types: Aichi virus 1, canine kobuvirus 1 (4), feline kobuvirus 1 (5) and murine kobuvirus 1 (6). The species Aichivirus B consists of three types: bovine kobuvirus 1 (2), ferret kobuvirus 1 (7) and ovine kobuvirus (8). The species Aichivirus C consists of a single type: porcine kobuvirus 1 (3). Recently, a distinct group of kobuviruses, designated caprine kobuviruses (CKOVs) was proposed as a new candidate species, Aichivirus D, within the genus (9).

Canine Kobuvirus (CKoV) is believed to have originated from the Aichi virus, 20-50 years ago. It belongs to Aichivirus A and is considered to be a genotype (CaKV type 1) distinct from murine kobuvirus (MuKV type 1) and human AiV (AiV type 1) (10). CKoV has a single strand (ss) RNA (+) genome and one large open reading frame (ORF) encoding a single polyprotein that is cleaved into 3 structural capsid proteins (VP0, VP1, and VP3) and 7 non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (4).

Several infections of viral origin are known to affect the

health and wellbeing of dogs (11).These include Canine Parvovirus (CPV), Canine Distemper virus (CDV), Canine Corona virus (CCoV), Canine Herpesvirus (CHV), Canine Adenovirus (CAV) and CKoV to name just a few. Out of these CDV causes a well-known, highly infectious viral disease with mortality in dogs, while current knowledge of kobuvirus infections in carnivores is extremely limited. CDV causes fever, vomiting, diarrhoea, respiratory symptoms, seizures and paralysis in the host. It is distributed globally with a broad host range, including many mammalian species of the families *Canidae*, *Mustelidae*, *Procyonidae*, *Ursidae* and *Viverridae* (12). The duration of the disease is based on the immune response to the CDV by the infected animal.

CKoV is very difficult to isolate and the diagnosis is mainly based on molecular methods. RT-PCR has been developed for the detection of CKoV in feces but further investigation is required for clarification of its pathogenesis (13, 14). The epidemiologic surveillance and genome characterization of CKoVs might help to clarify the global distribution of the virus and its possible association with enteric disease in dogs.

In the present study, we report the concurrent detection of two viral pathogens, Canine Distemper Virus and Canine Kobuvirus in a diarrhoeic sample of a Golden Retriever dog, which was brought to the Veterinary Clinical Complex of College of Veterinary Sciences, LUVAS, Hisar, for disease diagnosis and treatment. The dog showed clinical signs of gastroenteritis and was suspected to be suffering from Canine Distemper. Initial confirmation of the CDV infection was carried out using RT-PCR followed by conventional sequencing. Next-generation sequencing was used to characterize the viruses present in the diarrhea specimen. The study represents the first detection of Canine Kobuvirus in India from a diarrhoeic pet dog.

MATERIAL AND METHODS

For the molecular study, 50 faecal samples were collected from dogs brought to the Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS) clinics from October 2015 to September 2016. The cases presented for routine investigation or deworming and vaccination constituted the healthy control group. The faecal samples were collected by introducing sterile swabs into the rectum of dogs and were preserved in sterile PBS (1ml), vortexed and stored at -20˚C.

History, Physical and Clinical Observation

Fifty dogs up to the age of one year with clinical signs and history of vomition, diarrhea and nervous symptoms were included in the study. Complete history of the affected cases regarding the duration of illness, appetite, frequency of vomition and diarrhea, colour and consistency of vomitus and faeces, deworming and vaccination status, name and type of the vaccine administered, any previous treatment administered and other relevant data was recorded. The blood and serum biochemical parameters samples were fully analysed for complete hematological examination using automated Hematology cell counter (MS4s, Melet Schlosing Laboratories).

RNA Extraction and cDNA preparation

Commercially available live attenuated multi-component vaccine for CDV (Vencomax8®, Vencofarma, Londrina, Brazil) served as a positive control. The total viral (ss-RNA) was extracted from fifty diarrheic faecal samples suspected to be having canine distemper viral RNA as well as from the vaccine by using the combination of Trizol reagents and RNeasy plus universal mini kit (Qiagen, Germantown, Maryland USA). Samples after Trizol extraction were treated with DNase I (0.5 U/µl) at 23˚C for 15 min. This extracted RNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Waltham, Massachusetts, USA). The extracted RNA was further used for library preparation for Next Generation sequencing. cDNA was also synthesized from extracted faecal RNA samples using "Revert Aid first strand cDNA synthesis kit" (K1622, Thermo-scientific® Waltham, Massachusetts, USA) as per manufacturer's instructions and stored at -20°C till further use.

RT-PCR screening for the presence of CDV:

The faecal samples were screened for the presence of viral RNA by amplifying the cDNA in a thermocycler (Veriti, Applied Biosystems Waltham, Massachusetts, USA) prepared by conventional RT-PCR using published primer pair pCD /N/ (F) – ACA GGA TTG CTG AGG ACC TAT and pCD /N/(R)- CAA GAT AAC CAT GTA CGG TGC having an amplicon size of 287 bp targeting N gene of CDV. The reaction mixture and cycling conditions were used as per the methods described (15).

Sequencing of N gene amplicons of CDV:

The 287 bp amplicons obtained by PCR were purified using gel extraction kit (Qiagen, Germantown, Maryland USA) as per the manufacturer's instructions. The purified PCR amplicons CDV/IND/HSR/2016 isolate was sequenced directly using ABI 3130 XL Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) using CDV N gene specific PCR primers as sequencing primers. The contigs of forward and reverse nucleotide sequence obtained from this data were analysed using NCBI BLAST online software tool available on the internet (http://www.ncbi.nlm.nih.gov/BLAST/).

Whole genome sequencing and Library preparation of positive samples:

The whole genome viral library was prepared using10 ηg/μl concentration of total RNA. The cDNA library was prepared using Nextera XT DNA Library Prep Kit (Illumina Way, San Diego, CA, USA) using the standard protocol https://support.illumina.com/content/dam/illumina (support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-05.pdf). The purity and integrity of nucleic acid of samples were measured using Fragment Analyzer System (AATI, Newark, Delaware, United States). The Whole-genome sequencing was performed from the appropriate library prepared and sequenced with an Illumina MiSeq instrument (Illumina Way, San Diego, CA, USA) using 500 cycles, 250 paired end-sequencing protocol. The sequencing reads (Fastaq files) were assembled using the CLC workbench and *de novo* approach in the University of Minnesota using their in house developed NGS data analysis pipeline. The FASTQ files were analysed using in-house bioinformatics pipeline for trimming to remove Illumina adapters using Trimmomatic with a minimum quality score of 20 (v 0.39, https://github.com/usadellab/Trimmomatic). Then, host contamination was removed using bowtie2 (v 2.4.4, https://github.com/BenLangmead/bowtie2). The SPAdes (v3.15.2, https://github.com/ablab/spades) with k-mer values of 21, 31, 41, 51, 61, and 71 and the options --care was used for assembly of unmapped reads. Extracted contigs were analysed using BLASTx at NCBI to determine taxonomy. ORFs of assembled contig/genome were predicted using Vgas tool with default parameters (16, 17, 18, 19).

Phylogenetic analysis

Multiple sequence alignment of the whole genome of CKoV and partial N gene (287 bp) of CDV were carried out with the respective DNA sequences retrieved from NCBI database using Clustal W software implemented in Bio-Edit program (20). MEGA 6 software was used to construct a phylogenetic tree to show the genetic relatedness of CKoV and CDV from different origins (21). Bootstrap probabilities were calculated with 1,000 replicates. Neighbour joining (NJ) phylogenetic trees were constructed using default parameters of different nucleotide sequences of CKoV and CDV separately.

RESULTS

Canine Kobuvirus and Canine Distemper virus in domestic dogs in India

The domestic dogs suffering from gastroenteritis of up to one year of age which were brought to the Veterinary Clinical Complex of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana for treatment were investigated for the presence of viral origin gastroenteritis. In a previous study, it was found that dogs less than 12 months of age were at increased risk of developing these diseases due to non-vaccination or underdeveloped immune system. Therefore, dogs of one year of age or less were taken into this study (22, 23). Out of these samples, only one sample revealed whole genome of Canine Kobuvirus (CKoV) along with partial genome sequences of CDV.

History, clinical profile and laboratory findings of dog diagnosed with CD and CKoV gastroenteritis:

The only dog that was found positive for CDV and CKoV in this study was a two month old male golden retriever. This pup was born in tehsil Adampur, District Hisar, State Haryana. The dog was unvaccinated and born to a non-vaccinated mother. There was no history of movement of pup outside the country and any contact possible with the other dogs as it was separately housed with mother. However, the mother of dog was imported from U.S.A six years previously where CKoV was highly prevalent. The pup may have been infected from CKoV due to close contact with mother who might have acted as asymptomatic active carrier for this virus with low pathogenicity (10). The pup was showing signs of anorexia, lethargy, bilateral purulent

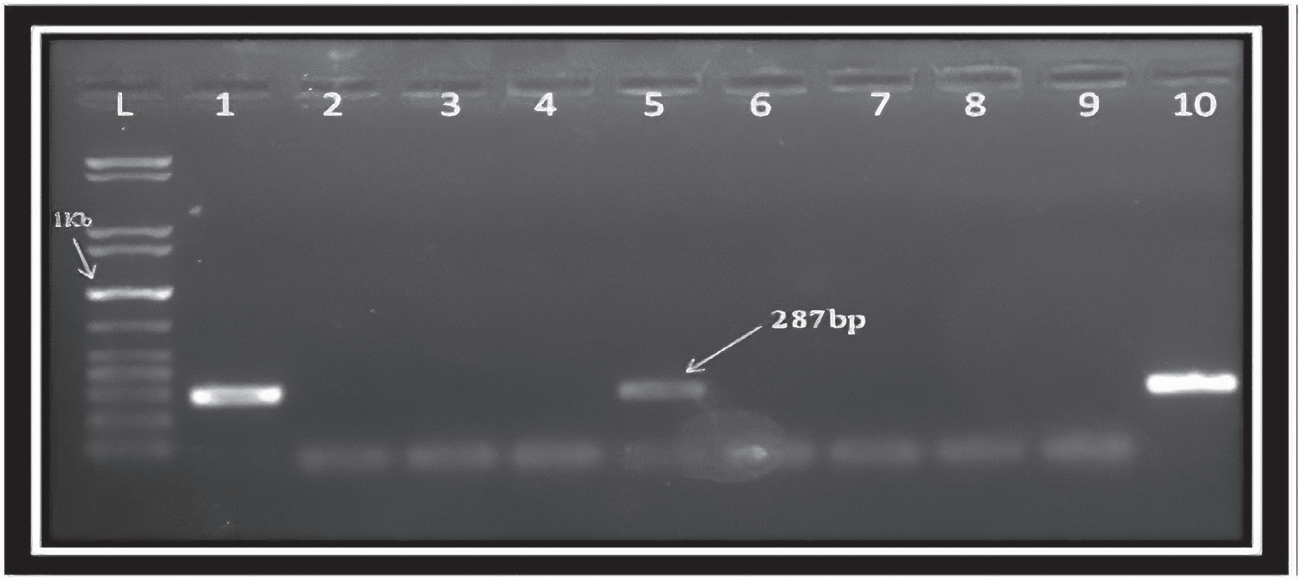

Figure 1. Agarose gel electrophoresis of 287 bp size PCR product of Canine Distemper virus positive sample. L: 1Kb ladder) Lanes 1 and 10: positive control) Lane 5: field sample (positive) Lanes 2 to 4 and 6 to 9: field samples (negative).

Table 1: Biochemical profile of a dog diagnosed with CD and CKoV gastroenteritis

Parameters	Dog affected with CD	Normal Reference range (Source: The Merck's Veterinary Manual, 11th edition)
AST (IU/L)	41.50	13-15
ALT (IU/L)	59.30	10-109
TP (g/dl)	4.80	5.4-7.5
Albumin (g/dl)	3.12	2.3-3.1
Globulin (g/dl)	1.68	2.7-4.4
BUN (mg/dl)	50.40	8-28
Creatinine (mg/dl)	1.68	0.5-1.7
Sodium (mEq/L)	127.50	142-152
Potassium (mEq/L)	4.35	3.9-5.1
Chloride (mEq/L)	102.10	110-124

ocular discharge, photophobia, fever, diarrhoea, dehydration, depression, pustules on abdomen and twitching of facial muscles. Haematological findings were suggestive of leucocytosis (29.50 10^3/µL) with relative neutrophilia (89%) and lymphopenia (9%) as compared to the mean control values for the respective parameters. The amount of haemoglobin (9.00g/dl), packed cell volume (29%) and total platelet count were also observed to be lower than the mean control reference values, which were 11.9-18.9 g/dL, 35-57% and 211-621x10^3/µL respectively. There were increased values of AST, ALT, BUN and serum creatinine, while a decrease in the values of total protein, albumin, globulin and serum electrolytes (sodium, potassium and chloride) in this dog (Table 1).

Phylogenetic analysis of canine distemper virus based on partial N gene sequences:

Rectal swabs were tested for the presence of CDV using RT-PCR as described in material and methods using published primers targeting N gene. The agarose gel electrophoresis revealed an expected band size of 287 bp (Figure 1). The partial sequence of N gene was submitted to GenBank under accession number MN128876.1. Phylogenetic analysis based on a partial N gene sequence of CDV was conducted by comparing this sequence with the commercial vaccine strain and other CDV strain N gene sequences available on GenBank. CDV strain from Hisar showed maximum nucleotide identity of 97% with other Indian strain (Accession Number MH536200.1) and 97.15 % with the CDV isolate from Hebei, China (Accession Number KC427278) (Figure 2). CDV strain from Hisar had 12 nucleotide differences with Rockborn strain over a sequence length of 287 bp. The field CDV sample had 95.8% nucleotide identity with the Rockborn strain of vaccine virus.

Table 2: Pairwise comparison of percent nucleotide identity of different genes of Indian CKoVs and worldwide distributed other isolates

Viruses (Country, Year)	Accession number	L gene	VP0 gene	VP3 gene	VP1 gene	2A gene	2B gene	2C gene	3A gene	3B gene	3C gene	3D gene
CKoV CH-1 (China, 2012)	JQ911763.1	95.53	94.93	93.58	88.78	98.20	95.96	95.62	96.13	95.06	97.61	97.02
CKoV CU_101 (Thailand, 2018)	MK201777.1	94.16	94.58	94.05	85.80	97.31	95.15	96.12	96.13	95.06	97.26	96.90
CKoV AH-1/CHN/2019, (China, 2019)	MN449341.1	94.16	95.01	93.58	85.70	97.01	95.15	96.12	95.42	95.06	96.58	97.64
CKoV 12D049 (South Korea, 2013)	KF924623.1	92.22	93.43	92.71	92.52	97.26	95.15	95.52	95.42	95.06	96.24	95.30
CKoV strain CU_249 (Thailand, 2018)	MK201778.1	95.14	93.96	93.58	86.07	97.90	94.95	96.02	96.13	93.83	96.24	97.64
CKoV_CE9_AUS_2012, (Australia, 2012)	MH052678.1	92.22	91.69	92.11	90.38	97.01	95.15	95.22	94.68	95.06	96.24	95.91
CKoV SMCD-59 (China, 2017)	MF062158.1	94.36	93.96	93.43	84.73	97.01	95.76	95.72	95.07	95.06	96.15	97.02
CKoV US-PC0082 (USA, 2011)	JN088541.1	92.55	91.34	92.56	92.27	96.11	93.54	94.52	94.37	95.06	95.12	95.41
CKOV CU_716 (Thailand, 2018)	MK201779.1	94.36	93.16	93.45	85.07	95.81	93.74	95.12	95.42	95.06	96.24	97.39
CKoVdog/AN211D/USA/2009 (USA, 2009)	JN387133.1	92.65	91.16	92.56	91.07	96.11	92.73	94.82	92.25	95.06	94.95	95.53
CKoV CaKoV-26 (Brazil, 2018)	MH747478.1	93.19	92.48	92.54	87.51	96.71	94.14	94.42	95.77	95.06	94.70	95.91
CKoV 1 isolate DD2 (Tanzania, 2015)	KM068048.1	93.58	91.16	92.69	89.76	94.91	92.73	92.94	94.37	90.12	94.02	95.29
CKoV S272/16 (Germany, 2019)	MN337880.1	92.02	90.99	93.75	89.90	94.61	92.32	93.63	96.13	95.06	94.92	95.66
CKoV 1 isolate B103 (Africa, 2015)	KM068051.1	91.63	90.90	91.94	89.05	96.41	92.73	92.25	92.61	95.06	94.53	95.04
CKoV UK003 (UK, 2013)	KC161964.1	94.94	92.65	93.90	91.21	95.81	91.52	94.72	93.29	95.06	95.30	96.40

Phylogenetic analysis of Indian Canine Kobuvirus based on whole genome sequence:

The next generation sequencing yielded major reads containing whole genome of Canine Kobuvirus (CKoV). The final sequence of CKoV (CKoV/IND/HSR/2016) was 7975 nucleotides long. The whole genome sequence obtained has been deposited in the GenBank database with accession no. MT610361. Phylogenetic analysis was conducted by comparing whole genome sequences of CKoV with other available sequences present in Genbank. The CKoV under study (CKoV/IND/HSR/2016) revealed 95% nucleotide identity with Canine Kobuvirus CH-1, Chinese strain (Accession Number JQ911763.1) and 94% nucleotide identity with Canine Kobuvirus strain CU_101 from Thailand (Accession Number MK201777.1) (Figure 3). This Indian strain has 371 base pair difference from the Canine Kobuvirus CH-1, Chinese strain. The CKoV under investigation was closely related to Aichivirus A, containing Kobuviruses isolated from dogs, bats, cats and humans. The Indian strain revealed 94.59%, 94.01% and 93.68%, nucleotide identity with Korean, Australian and USA strains, respectively. Based on whole genome analysis, the Indian strain (CKoV/IND/HSR/2016) clustered closely to the virus isolated from China, Thailand and differed from sub-cluster from the viruses from the USA, Brazil, Australia, Japan and Germany. On the basis of VP1 gene, this strain revealed, 92.54 %, 92.29% and 91.23%, nucleotide identity with Korean, USA and UK strains respectively. The pairwise comparison of other genes of Indian strain with other worldwide distributed viruses is given in Table 2.

Genetic analysis of Indian Kobuvirus based on whole genome sequence

The whole genome obtained for CKoV (CKoV/IND/HSR/2016) was 7975 nucleotide long and contains an open reading frame (ORF) of 7335 nucleotides (602-7936) encod-

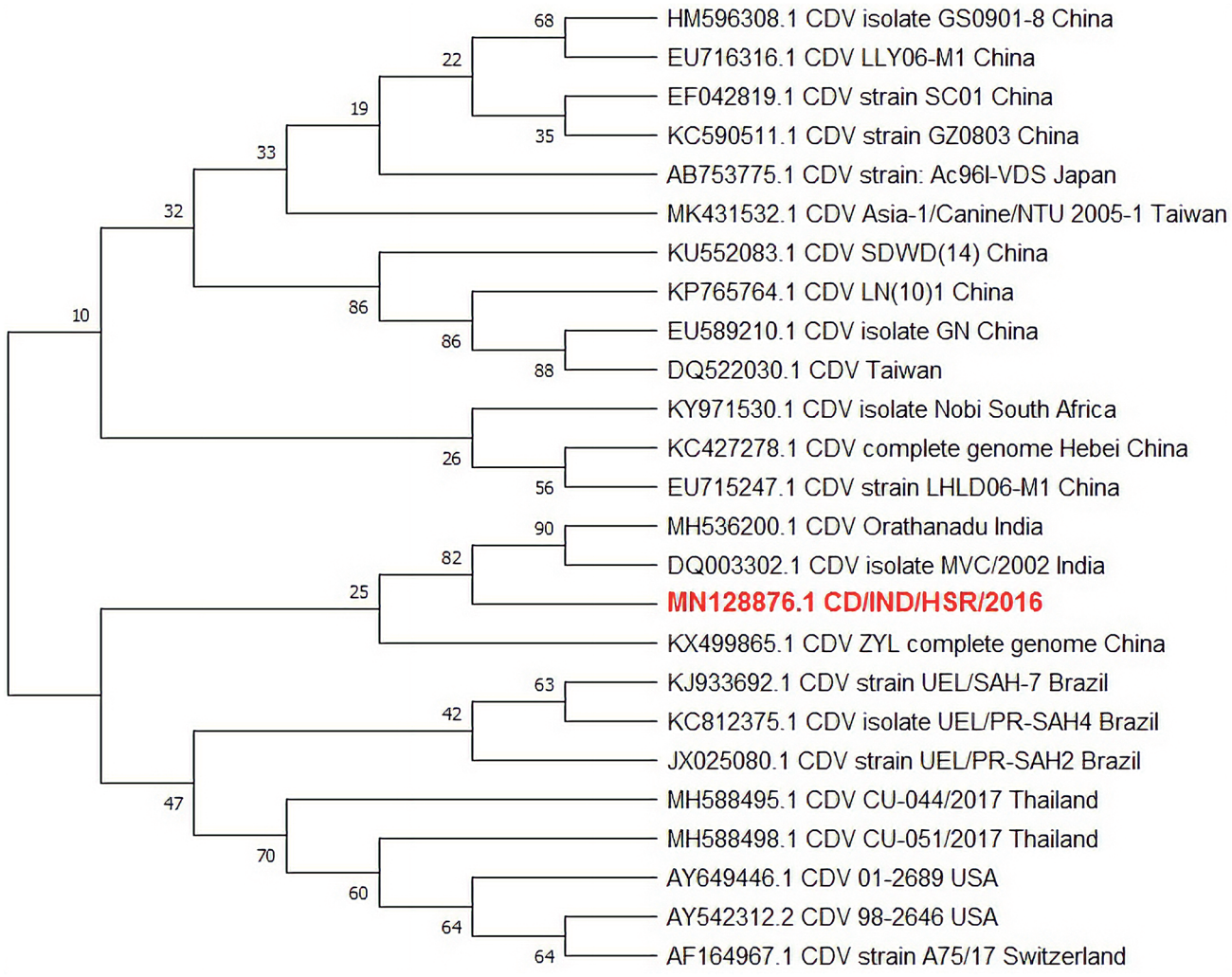

Figure 2. Phylogenetic analysis of CDV strain using the nucleotide sequences of the partial N gene of CDV. The Indian Kubvirus strain is indicated in red.

ing a putative polyprotein precursor of 2444 amino acids (aa). The full genome of CKoV (CKoV/IND/HSR/2016) had a high G+C content (58%) compared to some other kobuviruses (52–59%).

DISCUSSION

Viruses such as Parvovirus, Distemper virus, Coronavirus, Rotavirus, Adenovirus, Herpesvirus, Influenza virus and Parainfluenza virus have been reported as potential canine pathogens (24,25,26) as these are highly infectious viral pathogens and cause high morbidity and mortality in affected dogs. Diseases caused by CKoV infection in domestic dogs have remained unclear until now. Some workers have found that detection of CKoV RNA was not a major cause of diarrhoea in dogs (14) while CKoV was detected in outbreaks of acute gastroenteritis in canine shelters in the United States (4). The authors have also detected CKoV in dogs suffering from diarrhoea with the concurrent infection of canine coronavirus, canine parvovirus-2 and canine bocavirus (14, 27).

In the current study, the CKoV was detected in a domestic pup suffering from gastroenteritis of viral origin. During this investigation, the dogs having clinical signs of inappetence, diarrhea, vomition, nervous symptoms and dehydration were studied. Out of 50 screened samples for viral gastroentertitis infections, we found 25 dogs positive for CPV, 4 for CCoV and one for CDV. Routine diagnosis of CDV is done by IFA, ELISA and SN assays, virus isolation on canine cells but these are time consuming and do not

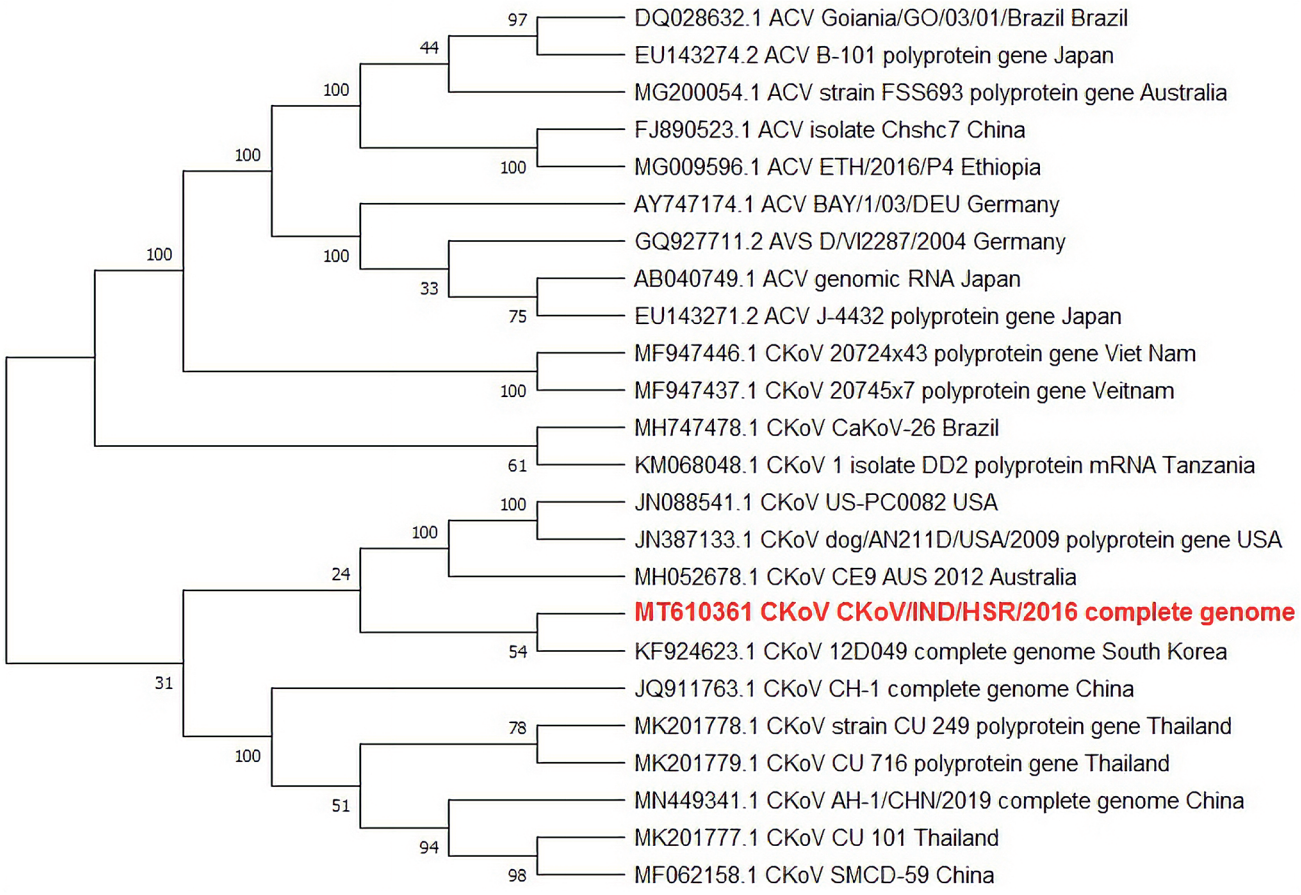

Figure 3. NJ tree of the completed genome of CKoVs. The phylogenetic tree was constructed by using MEGA v6.0 using P distance method. Values on branches represent bootstrap values. The Indian Kubvirus strain is indicated in red.

provide definitive diagnosis (28). Therefore, next generation sequencing analysis was done to obtain a definitive diagnosis and characterization of CDV genome that is circulating in dogs (29). In addition, NGS analysis from a single sample has the potential to identify multiple pathogens present in a single sample. The present study involving NGS of a sample showed the simultaneous presence of CKoV genome in that diarrheic faecal sample of a golden retriever dog which was also found positive for CDV in RT-PCR.

CKoV has been recognized in several countries in domestic dogs and wild animals. The prevalence of CKoV in domestic dogs has been reported to be 2.34% in Italy, 17.9% in China, 1.25% in UK and 19% and 13.2% of diarrheic and healthy dogs in Korea, respectively (10, 14, 30, 31). In Japan, 37.2% of diarrheic dogs and 48% of clinically healthy kennelled dogs were found to be positive for CKoV (32). There has been no prior report of infection of CKoV in dogs in India. The detection of CKoV in a young pup of two months of age corroborates with the previous observation that the CKoV may be frequently detected in younger dogs (32,33). In contrast to the earlier observations supporting the detection of CKoV in both diarrheic and non-diarrheic dogs (31, 32, 33), the dog found positive in this study for CKoV was suffering from gastroenteritis and also CDV infection. The findings of the present study are in accordance with the previous studies (10,30, 31, 32), which support that CKoV may also be the cause of enteric diseases. There are many viruses responsible for diarrhea either alone or in connection with other viruses (co-infection), which seems to be the case here.

NGS is the best indicator of the relationship between samples collected in different geographical regions and may help in understanding the antigenic differences between different biological samples. It could have implications in

vaccine differentiation studies to control a particular pathogenic agent (34). Phylogenetic analysis of CKoV showed that Indian strain (CKoV/IND/HSR/2016) is closely related to Chinese and Thai strains with 95% and 94% nucleotide identity in complete genome sequences respectively. The CKoV strain from India (CKoV/IND/HSR/2016) had a nucleotide identity of 94.2% with Thailand isolate, 94% with South Korean isolate and 93% with Brazilian isolates. It is genetically different from isolates of Ethiopia, Germany and Vietnam sharing only 78% nucleotide identity.

The full genome of CKoV encodes for leader protein L, capsid proteins VP0, VP3, VP1 and nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D (27). In this strain, the composition of different proteins were leader protein L (171 aa), capsid proteins VP0 (381 aa),VP3 (224 aa), VP1 (278 aa) and non-structural proteins 2A (111 aa), 2B (165 aa), 2C (335 aa), 3A (94 aa), 3B (27 aa), 3C (390 aa) and 3D (269 aa). On the basis of deduced amino acid sequence, this isolate also revealed 89 changes at the amino acid level at different positions when compared only with the reference polyprotein sequence (YP_009380518) (27). There were 2 aa change in L protein, 3aa in VP0, 1 aa in VP3, 1 aa in 2B, 2 aa in 2C, 3 aa in 3A, 1 aa in 3B and 6aa in 3C. There was the major change of 70 aa in VP1 protein depicting a mutation in Indian strain encoding for capsid protein. VP1 capsid viral protein has been observed as the most variable gene in various CKoVs identified in different countries (31, 33). Similarly, this isolate also revealed 70 amino acids at the reference protein sequence of VP1. Moreover, the VP1 gene of Indian strain (CKoV/IND/HSR/2016) has more nucleotide identity to USA, Korean and UK strains. Moreover, in this strain, putative proline rich region was present in the portion of VP1 gene, which has also been found in similar studies of different isolates from China and Thailand (33, 35).

On the basis of genetic analysis, there were 89 different amino acids distributed in polyprotein in the Indian strain (CKoV/IND/HSR/2016) which are not present in other worldwide distributed isolates. These unique amino acids will be beneficial for the detection of viral origin and development of strain specific diagnostics. Genetically, these changes in amino acids may have resulted from the mutation of the virus during the course of infection to overcome the immune system of animals.

In conclusion, the present study revealed the presence of CKoV along with CDV in the faecal sample of a young dog suffering from diarrhoea in India. This molecular characterization of the novel CKoV complete genome from India may help in studies related to molecular epidemiology, diagnostics development and vaccine development for these viruses. This is the first report of concurrent infection of CDV and CKoV in diarrheic faecal samples of dogs in India.

CONCLUSION

Concurrent viral infections in canine gastroenteritis cases are possible and may result in the greater severity of the disease. The diagnostic studies conducted herein can prove to be an important tool for studying molecular epidemiology of significant canine viral pathogens.

CONFLICT OF INTEREST

All authors have declared no conflict of interest.

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ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as described on the journal's author guidelines have been adhered to. This manuscript uses samples collected from dogs, which were clinically infected and brought for routine diagnosis and hence no ethical approval was required. A registered and experienced veterinarian collected the samples.

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