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Chicken Meat as a Source of Avian Influenza Virus Persistence and Dissemination

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Abstract: A study was conducted to investigate the persistence of Avian Influenza Virus (AIV) serotype H9N2 in the processed and frozen meat from chickens earlier exposed to this virus. For this purpose a group of chickens were experimentally infected with a field isolate (A/Chicken/Pakistan/NARC-02/2002) of AIV H9N2. After ten days post-inoculation, chickens were euthanized and their carcasses were cut into small pieces and stored at -20°C till further use. Subsequently, after every week selected pieces of frozen chicken were processed for isolation of AIV subtype H9N2 through embryonated egg inoculation. The data indicated that the AI virus was recoverable at different HA titres from various parts of chicken meat which includes neck, wings, breast, oscoxy, legs and bone marrow. It was possible to recover the AI virus using standard *in ovo* propagation techniques up to six weeks post-storage from most parts of the stored chicken.

Key words: Avian influenza virus (AIV), chicken meat, H9N2

Introduction

The major respiratory infections in humans and animals are known to be caused by different types of influenza viruses. The influenza viruses of type A include serotype H1 to H16 out of which H1, H2 and H3 are known to induce human flu. Over the past ten years some poultry specific Influenza viruses, belonging to sero-types H5, H7 and H9, are reported to be involved in causing infections in humans as well (Class *et al.*, 2000). High pathogenic forms of these viruses have been reported to produce heavy mortality in chickens, whereas, the low pathogenic forms induce drastic decline in egg production in laying hens. A number of studies carried out on low path Avian Influenza Virus (AIV) H9N2 have revealed that the virus is capable of causing high mortality in chickens previously immunosuppressed due to other biological/chemical or environmental stress (Bano *et al.*, 2003). Another study undertaken to determine the zoonotic potential of AIV H9N2 has revealed 95% homology between RNA sequence of H₅ and H₉ and has also shown seroprevalence of this virus amongst humans working in close proximity of birds such as farm workers (Cameron *et al.*, 2000).

As the AIV sub type H9N2 is known to share common genes with serotypes H5, H6 and H7 of AIV group, it becomes important to investigate the role of H9N2 in causing human and poultry infection. The study reported here was conducted to assess the ability of AIV H9N2 to persist in chicken meat, after slaughtering of infected chickens, which may contaminate the persons handling or processing chicken meat resulting in contaminating the environment for subsequent infections to human or poultry.

Materials and Methods

Virus and Chickens: An AIV subtype H9N2 isolate A/Chicken/Pakistan/NARC-02/2002 was obtained from the virus repository at the National Reference Lab for Poultry Diseases (NRLPD), National Agricultural Research Center (NARC), Islamabad. For this experiment 10 broiler chickens of four weeks of age were divided into two groups and reared in chicken-isolator at the Animal House facility of the NARC. One group (n = 5) was exposed intra nasally to H9N2 with a virus dose of 10⁵ EID₅₀. The chicks in the second group served as sham control. At 10 days post inoculation, chickens were slaughtered and their body parts packed separately in plastic bags. This included neck, wings, ribcage, breast, oscoxy and legs and stored at -20°C. At regular weekly intervals, these meat pieces from different parts of experimental chicken were processed for virus isolation by inoculating the processed samples into 9-days old embryonated chicken eggs as described below. Four days later, the allantoic fluid from the eggs was tested for HA activity.

Virus Isolation: To isolate AIV from chicken meat, 10 grams of frozen meat was homogenized in pestle and mortar to prepare 10% homogenate in Phosphate Buffer Salt solution (PBS) pH 7.2 containing 3x antibiotics (Penicillin - 2 x 10⁶ IU / liter, Streptomycin - 200mg / liter, Gentamycin-250mg/liter). The material was centrifuged at 800xg for 10 minutes at 8°C. The supernatant was filtered through 0.2 µm syringe filter and 0.2 mL per sample was inoculated into allantoic fluid of 9-day old embryonated chicken eggs. The eggs were incubated at 37°C for four days. Upon harvesting, the fluid was tested for HA activity. The HA positive fluid was treated and

Table 1: Recovery of AIV H9N2 from various parts of frozen chicken meat

Weeks Post-storage	----- Chicken meat portions showing Viral Presence -----					
	Neck	Wings	Breast	Osoxy	Legs	Bone Marrow
Weeks	----- n = 5* -----					
0 Week	4	5	2	0	4	5
1 st Week	4	5	2	0	4	5
2 nd Week	4	5	2	0	3	5
3 rd Week	3	4	0	0	3	5
4 th Week	3	4	0	0	2	5
5 th Week	0	0	0	0	2	5
6 th Week	0	0	0	0	2	5

*Indicates 5 individual chickens experimentally inoculated with AIV subtype H9N2. The infected meat samples were stored at 20°C and tested for the presence of H9N2 at different time intervals using embryonated chicken inoculation and HA procedure; #+ means the samples in each column tested positive for the presence of H9N2

neutralized with anti-sera to H9N2 for confirming the serotype of the recovered viruses. For this purpose an equal quantity of allantoic fluid and anti-serum were mixed in sterile tube at 4°C for one hour. The material was processed for HA activity and any negative HA was considered as the recovered H9N2 viruses being neutralized with the known anti-H9N2 antisera.

Haemagglutination Test (HA): The presence of AIV in the samples was determined using standard procedure of haemagglutination test (Hierholzer and Suggs, 1969). For this purpose 96 well U-shaped micro- titration plates were used. Two-fold dilutions of the sample in a total volume of 50 µL were made in PBS (7.2). To all the wells 50 µL of chicken RBCs (0.5% suspension in PBS) were added. The plates were incubated at 4°C for 30 minutes and HA activity was recorded.

Results

Table 1 shows the presence of AIV H9N2 in different parts of chicken meat frozen at variable time interval post-storage. At zero week (which is 10 days post AIV exposure) post storage (soon after slaughtering), the virus was detected in neck, wings, breast, legs and bone marrow tissues of the challenged chicks. Interestingly, none of the chickens yielded AIV in osoxy tissue at the start of the freezing process. Virus was readily detectable upto four weeks post-storage in neck and wing tissue but continuously upto six weeks in legs and bone marrow. None of the five non-infected control chickens showed any evidence of AIV presence in any of the tissue tested. Table 2 shows the geometric mean titers of AIV H9N2 as determined by HA. The neck, wings, legs and bone marrow tissue had a high starting HA titer whereas the breast tissue had very low levels and the osoxy did not reveal any positive titers at all. Overtime, at five weeks of storage and beyond, no HA titers were observed in all but legs and bone marrow tissues, suggesting an enhanced persistence of AIV in legs and bone marrow. Of all the tissues tested, the

bone marrow tissues showed much higher and persistent HA titers throughout the experimental storage period.

Discussion

Influenza viruses of type A have emerged as a zoonotic disease of great significance of recent times. Some of the major sub types in this regard include H5, H7 and H9 (Olsen *et al.*, 2002). In recent years, sub-type H7 and H9 have been incriminated in respiratory tract infections among humans in Holland and Hong Kong, respectively. H5N1 has been incriminated in more than 140 deaths during the past seven years in different countries in Europe and Asia (Guan *et al.*, 2004). The surveillance of live poultry markets in December 1997 in Hong Kong revealed that H9N2 influenza viruses were the most commonly isolated virus, marking up to 4% of infected poultry. The H9N2 virus is also reported to be endemic in many countries of Middle East and South East Asia. In Pakistan numerous H9N2 outbreaks involving laying hens and broilers have been reported (Naeem *et al.*, 2003). A study carried out on Pakistani isolate of AIV H9N2 revealed that this was closely related on the basis of NA sequencing to the isolates of H₉ which caused human infection in Hong Kong during 1997 (Hay *et al.*, 2000). These findings suggest that AIV H9N2 is a probable candidate for human infection in this part of the world, due to the close contacts of poultry and human in the prevalent environment, any re-assortment and emergence of new genetic variants of AIV is highly likely. In this scenario the present study was planned to identify different ways and means by which AIV H9N2 may persist in a population and can be transmitted through consumption and handling of chicken products by humans. This may eventually help in determining and subsequently eliminating the major reservoirs of infection among poultry.

The present study has revealed that AIV serotype H9N2 prevalent in local poultry population can cause viremia upon infection and persists in various body parts of the infected birds. The virus also resists continuous freezing for a period up to at least six weeks in chicken meat and bone marrow. Although, it was noted that the titers of the virus recovered from frozen meat declined significantly over a period of time, it was observed that AIV titers remained consistent in bone marrow in frozen chicken. This also reflects that despite being classified as a non-pathogenic virus, local isolates of H9N2 subtype has the capacity to cause systemic infection in chickens. It, therefore, may have the potential for transmission to humans through contact with products from infected chickens. A similar finding was reported by Tumprey *et al.*, 2002, where AIV sub type H5N1 was recovered from duck meat over a period of time post infection. The AIV H5N1 isolates from outbreak in South Korea when

Table 2: HA titer of avian influenza Virus from frozen chicken meat

HA Titers (GMT) of AIV H9N2 among different parts of frozen chicken*						
Weeks	Neck	Wings	Breast	Oscocy	Legs	Bone Marrow
0 Week	1024	980	118	0	948	960
1 st Week	870	850	70	0	301	860
2 nd Week	414	212	21	0	204	810
3 rd Week	55	112	0	0	45	340
4 th Week	145	24	0	0	26	180
5 th Week	0	0	0	0	14	680
6 th Week	0	0	0	0	3	414

* = Data in each column indicates the Haemagglutination titer of H9N2 in chicken meat samples at indicated sampling time (weeks) post storage

experimentally inoculated intra nasally caused systemic infection in chicken and quail and killed them within 24 h. The viral isolation from the organs and tissues revealed low virus titer in ducks than in the chicken and quail tissues (Lee *et al.*, 2005). In another study, Swayne and Beck (2005) isolated H₅N₂ and H₅N₁ subtypes of AIV from the respiratory and gastrointestinal tract of chickens. These low path AIV were not recovered from blood, bone marrow, breast or thigh muscles and the virus was unable to spread to other chickens. However the high path AIV isolate when experimentally inoculated in chickens caused systemic spread and isolation was done from blood, bone marrow, breast and thigh muscles. While the AIV has been isolated from tissues after experimental exposure, studies also have shown that AIVs such as H5N1 in meats from quail, chicken, ducks and geese if cooked as per Food Safety and Inspection Services (FSIS, USA) cooking standards, the temperatures such as 57°C to 61°C are sufficient to kill food-borne AIV (Thomas and Swayne, 2007).

The AIV H9N2 out break of 1997 in Hong Kong created awareness regarding Avian Influenza H₉ virus that could spread directly from poultry to humans. It is also apparent that it has a pandemic potential among humans, resulting from re-assortment between Avian and Human Influenza A viruses. The isolation of H9N2 from frozen chicken meat in this study suggests that uncooked infected chicken or its improperly disposed off waste could act as a source of AIV exposure to human and poultry. It has been previously reported that the Pakistani isolate H9N2 had close homology with the sequence of AIV H₉, earlier isolated from human infections in Hong Kong. There is also a report of 95% homology of H₅ genome with H₉ genome. This reflects the zoonotic potential possessed by H9N2. It therefore, becomes necessary to adopt appropriate measures for the proper control and eradication of H₉ from the local poultry population in the country. One of the parameters of effective control programs is to undertake virological and serological surveillance for H9N2 viruses in avian species and humans. This would lead to determine any change in the continuously mutating genome of AIV for avoiding any future risk to public health in this country.

Despite regular vaccination against AIV sub-type H9N2, the disease is still prevalent at vast scale throughout the country, both in layers and broilers where it causes heavy production losses in laying chicks and early chick mortality in broilers.

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