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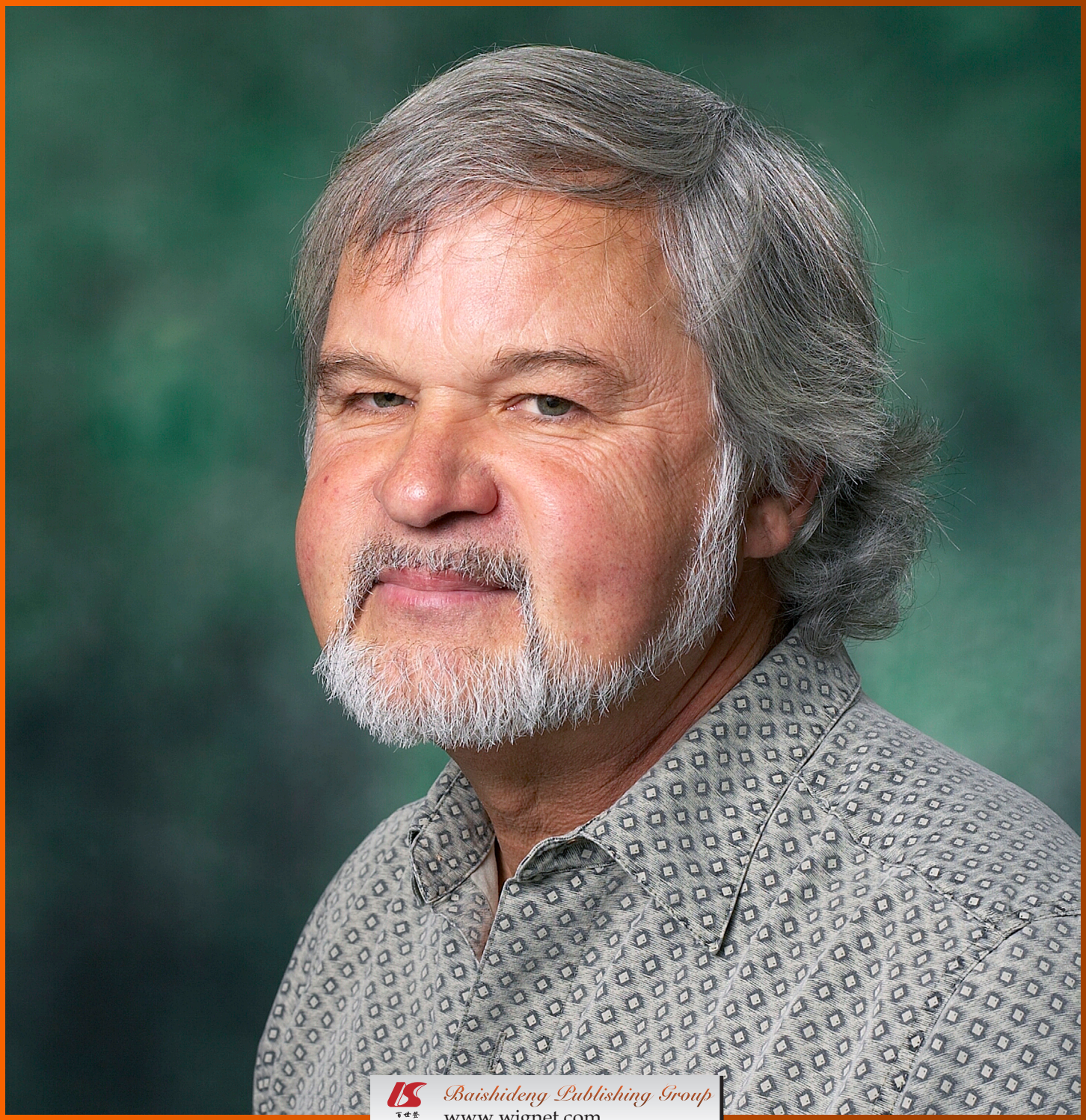

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MINIREVIEWS

Does prophylactic antidepressant treatment boost interferon-alpha treatment completion in HCV?

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Abstract

Depression is often a side effect of interferon-alpha treatment for hepatitis C, and is recognized as a cause for treatment discontinuation. When detected, antidepressant treatment begins promptly. In contrast to this rescue approach, prophylactic antidepressant treatment has been considered as a superior approach. While studies indicate that depression is lower with prophylaxis, no study has prospectively evaluated the degree that treatment completion might be boosted by the prophylactic strategy. A structured literature search was conducted to discover all trials of antidepressant prophylaxis for patients undergoing antiviral treatment for chronic hepatitis C. Selection criteria included: antidepressant prophylaxis study; report of depression treatment outcome; report of numbers discontinuing and reason for discontinuation (including any of the following: discontinuation data for medical side effects (*i.e.*, thrombocytopenia); discontinuation due to lack of antiviral response; discontinuation due to lack of antidepressant effect; discontinuation due to antidepressant side effects; discontinuation due to patient preference; discontinuation due to loss to follow-up; or unspecified discontinuation). Across the studies, total enrollees were determined for the prophylaxis arms and the rescue arms, and then, again across studies, those discontinuing for reasons other than lack of antiviral response or medical side effect were summed for each of these two arms. Twelve studies were discovered. One was a retrospective chart review, one was an uncontrolled trial, and ten were controlled trials. Discontinuation of antiviral therapy was not less common in the prophylaxis arms: of the 396 patients treated by the prophylaxis strategy, 47 (11.9%) discontinued; of the 380 patients in the rescue strategy, 45 (11.8%) discontinued. While the prophylaxis strategy seems to manage depression symptoms, it does not seem to boost treatment completion. Rescue was a very successful strategy when indicated. While antidepressant prophylaxis has benefit in antiviral treatment, it should not generally be valued for boosting the likelihood of treatment completion.

Key words: Depression; Therapy; Clinical; Psychiatry

Core tip: To inform clinical practice, this narrative review summarizes existing evidence regarding the degree that antidepressant prophylaxis boosts hepatitis C antiviral treatment completion compared to a rescue approach.

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INTRODUCTION

Although pegylated interferon-alpha may provide a sustained viral response from chronic hepatitis C infection[1,2],

this lengthy regimen is challenging to tolerate. Depressive symptoms, one of the more difficult side effects, can lead to discontinuation. Discontinuation rates for factors other than antiviral non-response range from 10% in well-conducted clinical trials[1,2] to 30% or more in clinical settings[3]. If depressive symptoms emerge, they must be clinically managed, including suspension of antiviral treatment as a last resort. Direct-acting antiviral agents may eventually supplant interferon-alpha/ribavirin regimens as standard of care[4], but interferon-alpha-based regimens have recently been re-affirmed as standards of care[5,6].

To reduce the threat of treatment-related depression, the idea of prophylactic depression treatment emerged[7]: when beginning interferon-alpha (and ribavirin) treatment, the patient would be started on an antidepressant with the goal of preventing, or attenuating, depressive symptoms. Initial case studies and case series noted success of this strategy. For example, antiviral treatment was restarted in a cohort of eight chronic hepatitis C patients who previously had discontinued due to emergent depressive episodes; all eight were able to fully complete the second course of treatment[8]. A precedent for this strategy was noting the success of antidepressant prophylaxis for interferon-alpha treatment of malignant melanoma[8,9].

Compared to prophylaxis, traditional practice can be termed "rescue" when depressive symptoms emerge in a patient undergoing antiviral treatment, depression treatment is quickly initiated so that those symptoms can be managed. The advantage to the prophylactic strategy is that depression and the threat of discontinuation can be avoided; the advantage to the rescue strategy is that patients are not unnecessarily treated, and so are not experiencing the additional treatment burden and side effects. Antidepressants may have quite adverse side effects in some patients, including retinal or gastroenterological bleeding[10,11]. Thus, clinicians are faced with a challenging clinical management strategy where risks and benefits must be considered.

Can prophylactic antidepressant treatment boost interferon-alpha treatment completion in patients with chronic hepatitis C virus (HCV)? No study has prospectively answered this question. This review has been conducted to discern an answer by reviewing antiviral therapy discontinuation data reported in trials evaluating the efficacy of antidepressant prophylaxis for managing depression. This review is presented in order to enhance the evidence available for clinical decision-making.

LITERATURE SEARCH

A Pubmed literature search was designed to discover trials that might have the data necessary to assess relative treatment completion between prophylaxis arms and control arms. A set of search terms was developed to capture studies relevant to hepatitis C. This included: "hepatitis", "HCV", "Hep C", "Hep-C" and "chronic hepatitis C". This was crossed with each of two other sets. The first was a set to capture depression-related studies: "depression", "depressed", "depressive", "psychiatric", "mental". The other was a set to capture prophylactic strategies: "prophylaxis", "prophylactic" or "prevention".

From this search, all study titles would be reviewed to detect promising abstracts. All promising abstracts would be read, and likely studies would be pulled and assessed for necessary information. References of those studies would be checked manually.

The necessary information for selection into this review was established as the following: patients with chronic hepatitis C who were candidates for interferon-alpha treatment (whether including ribavirin or not, as this treatment strategy emerged as the prophylactic strategy emerged); recognized treatment regimen (*i.e.*, interferon-alpha with ribavirin); no concurrent treatment such as for human immunodeficiency virus, since symptoms and treatment side effects would be significant confounders; at least two study arms where one included prophylactic treatment with an antidepressant, whether open-label or blinded, and the other is a control arm, whether placebo-controlled or not; sustained treatment of at least 8 wk in order to observe emergence of depressive symptoms from interferon-alpha and assess differential depression response between arms; and data on the numbers of patients in each arm that discontinued, or were lost to follow-up, for reasons other than medical side effects (thrombocytopenia, *etc.*) or non-response to antiviral therapy. Thus, the discontinuation group of focus would be those who medically could have completed treatment but discontinued for a reason other than a medical reason. To the degree that discontinuation reasons, such as psychiatric side effects, would be specifically reported, these would be tabulated and compared between the intervention-arm participants and the control-arm participants. The reporting of discontinuation for psychiatric reasons, specifically, was thus not an inclusion criterion.

For each eligible study, the number of patients discontinuing would be noted for each of the arms of the study. A descriptive analysis would be developed based on those results. The goal would be to describe the degree, if any, that antiviral treatment completion might be superior for the prophylactic strategy, compared side-by-side with the rescue strategy. Since the data sources for this study consisted of previously-published research studies, ethics approval for this narrative review was not sought from an institutional review board.

SEARCH RESULTS

For the "prophylaxis" search term set, "pretreatment" was soon discovered as a synonym, so this was added to that set. The "prophylaxis" set returned 1302661 abstracts; the "hepatitis C" set returned 184063 abstracts; and the "depression" set returned 869174 abstracts. The intersection of these three sets returned 419 abstracts. Titles of all were reviewed, leading to a set of 38 abstracts to review. This led to a set of 12 studies[8,12-23] in which the prophylactic strategy was evaluated, and discontinua-

Table 1 Study characteristics and discontinuation data: Antidepressant prophylaxis for interferon-alpha treatment of hepatitis C virus

Study	Leading exclusion criteria	Prophylactic intervention	Medication run-in period	Randomized: Yes/No	Blinded: Yes/No	Follow-up point, in wk[1]	Discontinued/Total in Arm:[2]	
							Prophylaxis	Rescue
Schaefer *et al*[12]	Psychiatric history, interferon treatment	Escitalopram	2 wk	Yes	Yes	24/48	2/90	3/91
Klein *et al*[13], Klein *et al* [14][4]	Active psychiatric disorder in the recent six months	Citalopram	3 wk	Yes	Yes	12	3/29	1/30
de Knegt *et al*[15]	Current Axis I disorder or current psychiatric prescription	Escitalopram	2 wk	Yes	Yes	24	2/34	2/37
Morasco *et al*[16]	Recent 3 mo psychiatric disorder	Citalopram	2 wk	Yes	Yes	24	N/A[3]	N/A[3]
Diez-Quevedo *et al*[17]	Active psychiatric disorder in the recent two months	Escitalopram	2 wk	Yes	Yes	24/48	4/51	6/48
Liu *et al*[18][5]	Alcohol use during treatment	Specific antidepressants not reported	Not reported	No	No	24/48	11/23	2/25
Neri *et al*[19]	Substantial psychiatric history, interferon treatment	Individual, family, and marriage counseling	N/A	Yes	No	24	14/106: 9/106 for lack of compliance, 5/ for psychiatric reasons	28/105: 11/105 for lack of compliance, 17/105 for psychiatric reasons
Gleason *et al*[20]	Depression not yet in remission	Escitalopram	4 wk	N/A	N/A	24/48	2/10	No control group
Morasco *et al*[21]	Active psychiatric disorder in recent six months	Citalopram	4 wk	Yes	Yes	24	3/13	3/15
Raison *et al*[22]	Psychiatric disorder or prescription within recent six months	Paroxetine	2 wk	Yes	Yes	24	0/18	6/18
Kraus *et al*[8]	Active substance abuse	Paroxetine or citalopram	3 wk	No		15	0/8	Not reported for control group of 9
Schaefer *et al*[23]	Current or recent psychiatric diagnosis or prescription	Citalopram	2 wk	N/A	N/A	24	0/14	0/11
						Totals:	47/396	45/380

[1]If 24/48, then treatment period accorded to interferon alpha regimen according to genotype, typically 24 wk for genotypes 2 and 3, and 48 wk for genotypes 1 and 4; [2]Denominator is total, including those lost to follow-up, who could have dropped out due to psychiatric reasons, and excludes those without this possibility, which would include the following: non-response to antiviral therapy, drop-out after randomization but before beginning antiviral therapy, or medical adverse events such as thrombocytopenia; [3]Three of 39 altogether discontinued antiviral therapy, but study report did not distinguish between active treatment and placebo for medical or other discontinuations; [4]2008 study was a trial design report, and 2012 was poster of initial results recently presented at a conference; [5]Retrospective observational chart review study; analysis limited to the two of four study arms that constitute a prophylaxis *vs* rescue comparison; 19 of the 23 in the prophylaxis arm were already taking antidepressants and the remaining 3 were started prophylactically before starting antiviral therapy.

tion data were reported.

These studies are listed, with relevant study characteristics, in Table 1. All but one were prospective trials; one was a retrospective chart review study that composed a cohort of patients who were taking an antidepressant before the initiation of interferon-alpha treatment, and composed a control group of patients who required some kind of psychiatric treatment during interferon-alpha treatment. For the sake of completeness, this chart review study was included. One of the 12 studies (Gleason *et al*[20], 2007), among the first chronologically, did not have a control group; this study simply investigated treatment completion when a prophylactic strategy was trialed. This was included for completeness. For one study, the manuscript reporting the preliminary study design was available, and results have just recently been presented as a poster at a scientific conference; it is assumed that a more complete analysis will be forthcoming. For the sake of completeness, results based on this conference poster were included.

Clinical Interventions

All studies were conducted in the era of prescribing ribavirin along with interferon-alpha. Nearly all were conducted in the era of pegylated interferon, with the exception of some of the earlier-initiated participants in the Morasco *et al*[21] (2007) and Raison *et al*[22] (2007) studies. Likewise, antidepressant dosages were normative, with typical strategies for increasing or augmenting dosage when clinically indicated, and typical medication switching strategies when clinically indicated. All studies used antidepressants from the selective serotonin re-uptake inhibitor class, including paroxetine (one study), paroxetine or citalopram (one study), citalopram (five studies), and escitalopram (four studies). This usage followed the pattern of Food and Drug Administration approval and clinical

adoption of these drugs, with paroxetine favored in earlier studies, citalopram favored in the studies conducted in the middle of this time span, and escitalopram favored in later studies. A range of strategies were used to assess depression level before and during treatment. These generally included: standardized clinical interview, clinical interview, a depression questionnaire, or combination. In some studies, patients could be started on antiviral therapy even if some level of depressive symptoms was present.

Clinical outcomes

The overwhelming majority of patients were able to complete interferon-alpha treatment. Sustained viral response results were in line with other well-managed intervention studies using interferon-alpha and ribavirin (*e.g.*, approximately 40% sustained viral response for those with genotype 1, approximately 75% for those with genotypes 2 or 3). Some patients failed to show a treatment response, and so interferon-alpha was discontinued due to lack of response. Some patients had treatment-related adverse events, such as thrombocytopenia, requiring discontinuation of therapy. To the degree that these data were available, the current study did not include these patients in the denominator at risk of discontinuing due to psychiatric difficulties, since they had discontinued due to medical reasons. Patients who were lost to follow-up or discontinued for other preference or discretionary reasons, or for unidentified reasons, were included in the numbers of patients who discontinued treatment for some reason other than antiviral non-response or medical side effect. This strategy was chosen because it can be challenging, especially from limited data included in published studies, to determine the leading reason for discontinuation or loss to follow-up, and the clinical question is whether prophylaxis boosts study completion.

Generally, providing antidepressant treatment resulted in amelioration of depressive symptoms. For the groups receiving antidepressant treatment prophylactically, average levels of depressive symptoms, or the portion of patients with an emergent depressive disorder, were lower in those receiving prophylactic treatment *vs* rescue treatment. Generally, problems with depression were worse for those at baseline with any depressive disorder history, or with higher initial depression severity.

Despite the clinical efficacy of antidepressant prophylaxis in controlling depressive symptomatology, there seemed to be no indication that the prophylactic strategy boosted treatment completion rates compared to the rescue strategy. Table 1 presents these data by study, including a summation of the total number of patients in the denominator, at risk for discontinuation, for both prophylactic and rescue arms, and the number for both arms that discontinued therapy. Of 396 patients in the prophylaxis arms altogether, who did not discontinue due to medical adverse events or clinical non-response, 47 (11.9%) discontinued interferon treatment before a recognized stopping point (*e.g.*, 24 or 48 wk); of 380 patients in the rescue arms, 45 (11.8%) discontinued interferon treatment. There was no overall statistical difference when tested by Chi-Squared test with Yates' correction (χ^2 = 0.00, P = 0.99).

One study (Raison 2007) seemed to yield a desired effect for prophylaxis: none of the 18 prophylaxis patients discontinued, while 6 of the 18 rescue patients discontinued. A review of this study in the context of other studies did not reveal any clear aspect of study design, measurement, or sampling that would indicate an explanation for this divergent result from the other, similar studies.

The Liu *et al*[18] study (2010) had greater discontinuation in the prophylaxis arm, but the psychosocial intervention used in this study, close monitoring and various counseling modalities, and psychopharmacotherapy only in certain cases where this psychosocial intervention was not successful, was very different from the other studies. Aside from this differential in discontinuation, the psychosocial intervention used in the Liu *et al*[18] study otherwise was successful in managing psychiatric symptoms, and doing so with less dependence on psychopharmacotherapy, compared to the usual care arm with rescue psychopharmacology. In this Liu study, with a psychosocial strategy for prophylaxis rather than psychaopharmacotherapy, the number of patients experiencing severe psychiatric symptoms was lower in the intervention group, with five meeting this criterion, *vs* 17 in the control group. Psychiatric symptomatology at less severe levels, likewise, was less frequent for the intervention arm compared to the control arm, with only six of the intervention patients eventually receiving antidepressant treatment compared to 19 in the control arm.

There were nine studies with data that permitted a Fisher's Exact Test to test whether the discontinuation rate differed between prophylaxis arm and rescue arm. Of these nine, only four had results that were statistically significant. Three modestly favored prophylaxis. These were: Diez-Quevedo *et al*[17] 2010 (7.8% discontinuation in prophylaxis arm, 12.5% rescue arm, Fisher's P = 0.02), Neri *et al*[19] 2010 (8.5% discontinuation in prophlylaxis arm, 10.5% discontinuation in rescue arm, Fisher's P = 0.02), and Raison *et al*[22] 2007 (0.0% prophylaxis arm, 33.3% rescue arm, Fisher's P = 0.02). The one study favoring rescue was Liu *et al*[18] 2010 (47.8% discontinuation in prophylaxis arm, 8.0% discontinuation in rescue arm, Fisher's P = 0.02). With five studies having no statistical difference in discontinuation, three favoring prophylaxis by varying portions, and one favoring rescue by a strong portion, there seems to be no consistent pattern favoring either strategy.

Since these studies were focused upon the presence and severity of depressive symptoms, but not on reasons for failure to complete a full course of therapy, reasons for not completing therapy were not systematically reported, and those reporting did not use consistent criteria. For those that did report, the stated reasons for discontinuation are listed in Table 2. Predominant reasons for not completing therapy included: Lost to follow-up, psychiatric side effects, and non-adherence. These reasons are likely quite overlapping, such as a person

Table 2 For studies reporting discontinuation data, number discontinuing interferon-alpha therapy, and reason for discontinuation, summed across studies

Reason for discontinuation	Prophylactic arm	Rescue arm
Lost to follow-up	21	19
Psychiatric side effects	8	18
Non-adherence	11	14
Did not complete therapy	3	3
Noncompliant or loss to follow-up	0	6
Other side effects	0	4

choosing to fail to continue in treatment due to psychiatric symptoms.

DISCUSSION

Emergence of depressive symptoms is a challenging side effect when treating chronic hepatitis C with interferon-alpha. Rates of depression may be as high as 30% or more. It has been established that monitoring patients for the emergence of depression, and rescuing those in whom depression emerges, is a successful strategy for limiting treatment discontinuation or poor adherence. Because of this high incidence of treatment-related depression, the idea of prescribing an antidepressant prophylactically to all patients at the initiation of antiviral therapy is attractive. This search revealed 12 studies that have evaluated the benefits of prophylactic treatment. From these studies, it is clear that prophylactic treatment serves to reduce the emergence of depression, and serves to manage the level of depressive symptomatology.

This review was undertaken to investigate the degree that the prophylactic strategy might boost treatment completion. There is no clear indication that the prophylactic strategy generally serves to boost treatment completion, compared to a monitor-and-rescue strategy. Where noted, nearly all patients in the rescue arms were successfully rescued from the emergence of depression. Review of study parameters does not suggest any treatment strategy or patient profile where prophylaxis yields a boost in treatment completion.

Advantages to prophylaxis are the superior management of depression during treatment in some portion of patients. This advantage needs to be weighed against the negatives of this strategy, which include the increased treatment burden on the patient, increased cost, and the risk of adverse events from the antidepressant. Two of the reviewed studies indicate some likely applications for prophylaxis. The study by Schaefer *et al*[12] (2005) demonstrated lower rates of treatment-related depression in the prophylactically treated arm, compared to the arm with no prophylaxis, in a cohort of patients with chronic hepatitis C who also had a history of a mental disorder (predominantly affective and dependence disorders) but with no active symptomatology and not currently receiving any psychiatric medication. The Kraus *et al*[13] (2005) study demonstrated successful interferon-alpha retreatment with antidepressant prophylaxis for a cohort of patients who had previously discontinued interferon-alpha treatment due to the emergence of depressive symptoms, while the control arm experienced, on average, even higher depressive symptom levels in the second attempt at interferon-alpha treatment (possibly due to the use, for all, of pegylated interferon-alpha in the second but not first treatment attempt). So, certain subgroups with recognized psychiatric difficulties may benefit from antidepressant prophylaxis.

While psychopharmacology is effective for managing depression in interferon-alpha treatment of hepatitis C, it is interesting to note the positive results of the Liu study, with a psychosocial intervention including individual counseling, family counseling, and couples counseling. The exact design of this intervention was not reported, such as how counseling needs were discovered, or data on the number of sessions delivered, or the specific clinical issues addressed, or whether any component included comprehensive chronic illness management training (disease education, treatment education, stress management, physician-patient communication skills, *etc.*), which has been shown to improve treatment adherence along with health-related quality of life.

Why didn't the prophylaxis approach have superior treatment completion, along with superior depression management, compared to rescue approach? It is possible that, in these trials, the rescue strategy worked as well as prophylaxis because clinical trials often have clinical management practices (answering patient questions, establishing clear lines of communication, systematic symptom monitoring, recruitment of motivated patients) that is stronger than usual care. If this is the case, then those delivering interferon-alpha treatment for chronic hepatitis C should be sure to parallel the symptom monitoring strategy of these trials. The monitoring of depression is a topic that has already been covered well in the literature concerning antiviral therapy, and has long been incorporated into treatment guidelines. The results of the Neri *et al*[19] (2010) study support this possibility: strong psychosocial monitoring led to better affective symptom control, with only a small portion of that advantage due to the use of antidepressants. At the same time, it is valuable to note that, in the Liu *et al*[18] (2005) study, interferon-alpha treatment conclusion or discontinuation led to a reduction in the emergent depressive symptom levels seen, leading the authors to conclude that "depression was specifically related to IFN therapy".

One indirect benefit of antidepressant treatment may be the management of treatment side effects other than psychiatric side effects. Raison *et al*[22] (2007) found stronger completion rates in the prophylaxis arm, and this was noted as being related to lower antiviral side effect difficulties. The study by Diez-Quevedo *et al*[17] (2010) also noted lower levels of antiviral side effects in those receiving antidepressants. Antidepressants are used in a range of clinical indications beyond depression, such as management of pain and management of fibromyalgia symp-

toms. In antiviral therapy, antidepressants may somehow reduce a range of symptoms. This could explain an unusual finding regarding depression in a larger hepatitis C study[24] that used a rescue strategy for emergent depression: while depression emerged for 90 patients in this study of nearly 400, discontinuation rates were lower for those patients (6%) than for those in whom no depression emerged (15%). The antidepressant intervention, or the related social support experienced in the course of clinical response, may have served to ameliorate the experience of treatment side effects. Data were not sufficient in the studies reviewed here to investigate more fully the possibility that antidepressant treatment in antiviral treatment may ameliorate antiviral-related side effects.

Another treatment characteristic suggesting that prophylaxis has limited clinical benefit was the necessity of monitoring and rescuing patients in the prophylaxis group, as well as the rescue group. In the de Knegt *et al*[15] study (2011), with 40 patients in the escitalopram group and 39 in the placebo group, four in the prophylaxis group needed rescue (increase or augmentation of dose, or new medication) while seven patients in the placebo group needed rescue depression treatment. In the Schaefer *et al*[23] (2012) study, three in the prophylaxis group needed rescue by another antidepressant, while 16 in the rescue arm required rescue. In the Morasco *et al*[21] (2010) study, approximately 30% in each arm had to have medication dosage adjusted, with some of those in the prophylaxis arm entering "rescue" treatment. This need to monitor and adjust pharmacotherapy is a limit to the treatment efficiency to be gained by prophylaxis; prophylaxis does not reduce the necessity of monitoring patients for the emergence of depression symptoms, and so does not greatly lighten the task of clinical care required to manage depression.

Because the influences of cytokines upon the central nervous system are quite varied, it is not quite clear how interferon-alpha causes depression in some patients. Pro-inflammatory cytokines can experimentally induce "sickness behavior" in non-human animals. It is hypothesized that this malaise might serve a valuable function: when the body needs to fight off infection, it is advantageous to have a healing period of increased sleep, lower activity level, and lower appetite; pro-inflammatory cytokines promote inflammatory responses, and also may simultaneously be registered in the brain, leading to the coincident sickness behavior[25]. Research in humans has revealed that interferon-alpha has an array of effects in the central nervous system, and elevated cytokine activity, especially tumor-necrosis factor-alpha and interleukin-6 can be noted in some portion of cases of major depression[26,27]. Further, serotonin-acting antidepressants have an effect upon tumor-necrosis factor-alpha and interleukin-6, as well as other inflammatory markers[28].

Providers should be clear about desired purpose when considering prophylactic antidepressant for hepatitis C patients about to begin antiviral therapy. Antidepressant prophylaxis does not seem to boost treatment completion, so other goals, such as managing depression, should be clarified when considering the strengths and weaknesses of this strategy. Discontinuation of interferon-alpha for chronic hepatitis C is a great treatment challenge, and anything that interferes with completion of treatment should be well investigated.

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MINIREVIEWS

Impact of PRRSV on activation and viability of antigen presenting cells

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases of swine industry. The causal agent, PRRS-virus (PRRSV), is able to evade the host immune response and survive in the organism causing transient infections. Despite all scientific efforts, there are still some gaps in the knowledge of the pathogenesis of this disease. Antigen presenting cells (APCs), as initiators of the immune response, are located in the first line of defense against microorganisms, and are responsible for antigen recognition, processing and presentation. Dendritic cells (DCs) are the main type of APC involved in antigen presentation and they are susceptible to PRRSV infection. Thus, PRRSV replication in DCs may trigger off different mechanisms to impair the onset of a host effective immune response against the virus. On the one side, PRRSV may impair the basic functions of DCs by regulating the expression of major histocompatibility complex class Ⅱ and CD80/86. Other strategy followed by the virus is the induction of cell death of APCs by apoptosis, necrosis or both of them. The impairment and/or cell death of APCs could lead to a failure in the onset of an efficient immune response, as long as cells could not properly activate T cells. Future aspects to take into account are also discussed in this review.

Key words: Porcine reproductive and respiratory syndrome; Antigen presenting cells; Dendritic cells; Immune response; Major histocompatibility complex class Ⅱ; CD80/86; Cell death; Apoptosis

Core tip: Porcine reproductive and respiratory syndrome virus (PRRSV) is able to evade the host immune response and survive in the organism causing transient infections. PRRSV interacts with antigen presenting cells, specifically with dendritic cells, causing a regulation of major histocompatibility complex class Ⅱ and/or CD80/86 and cell death by apoptosis and/or necrosis.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS-virus (PRRSV)[1,2]. This virus belongs to the genus Arterivirus[3] and has a high genetic variability[4]. Two genotypes of PRRSV can be distinguished: PRRSV-1, which comprises strains from Europe; and PRRSV-2, which includes strains from America[3]. Indeed, PRRSV-1 is subdivided into three different subtypes, subtype 1, which includes strains from Western Europe, and subtypes 2 and 3, which comprise strains from

Eastern Europe[4]. Moreover, PRRSV-1 strains can also be classified in accordance with the capability to induce different patterns of tumour necrosis factor α (TNF-α), interleukin-10 (IL-10) after infection of peripheral blood mononuclear cells, porcine alveolar macrophages, peripheral blood SwC3$^+$ cells and bone marrow dendritic cells (BMDCs) into: IL-10$^+$-TNF-α$^+$, IL-10$^-$-TNF-α$^+$, IL-10$^+$-TNF-α$^-$ and IL-10$^-$-TNF-α$^-$-inducing strains[5].

Host immune response against the virus is weak and erratic and fails to control PRRSV. Different studies point out that type 1 interferons are insufficiently produced, which has been related to an inhibition of interferons by nonstructural proteins 1α, 1β, 2, 11, as well as, N protein[6-9]. Indeed, proinflammatory cytokines are also mildly produced[7,10,11] being associated nonstructural protein 2 to a decreased release of IL-1β and TNF-α[12,13]. Some *in vitro* evidences in the literature also point out that PRRSV can induce a suppression of NK cells[14]. Although PRRSV may induce the induction of cytotoxic T lymphocytes[15], these cells seem to suffer an impairment to exert their cytotoxic activity to PRRSV-infected macrophages[16]. Furthermore, the number of interferon-γ-secreting cells is not enough to control PRRSV[17-20] and neutralizing antibodies (NAs) are delayed and not produced in a vast extent[17]. Two to four weeks after infection, NA-response takes place, resulting in very low titers (1/32-1/64 or even lower)[17,18,20,21].

Antigen presenting cells (APCs) are located in the first line of defense against microorganisms attack. These cells recognize, process and present antigens to T cells in order to trigger an effective immune response[22-25]. While B cells can directly recognize antigens by means of its B cell-antigen receptor, T cells need the involvement of different molecules through two mandatory signals. The first signal consists on the binding between the T-cell antigen receptor (TCR) and the major histocompatibility complex class Ⅱ (MHC-Ⅱ) molecule. For the second signal, the CD28 molecule from T cells interacts with co-stimulatory molecules (CD80/86) from APCs. The correct linking of these molecules in the presence of antigens will suitably activate T cells[26,27].

Dendritic cells (DCs) are the main type of APC involved in antigen presentation. However, macrophages and B cells, although less efficiently, can also act as APCs[26,28-30]. Interestingly, it has been shown that different types of DCs and macrophages can suffer PRRSV replication *in vitro*[31-37]and *in vivo*[38-43]. However, in an *ex vivo* experiment, Loving *et al*[44] showed that lung-DCs were not permissive for PRRSV infection. A reasonable explanation for this result is that these lung-DCs could lack the receptors that PRRSV uses to go into the cell (*i.e.*, CD163, sialoadhesin, heparan sulphate)[45], while other types of DCs conserve these receptors. Furthermore, PRRSV replication directly impairs the basic functions of infected macrophages, including phagocytosis, antigen presentation and production of cytokines, and also induce cell death[46]. Therefore, changes in the number of APCs and/or a downregulation on the expression of MHC-Ⅱ and CD80/86 may lead to suppose an impairment in the onset of an effective immune response against PRRSV.

Other strategy followed by PRRSV to evade the host immune response might be the induction of cell death of APCs by apoptosis, necrosis or both of them[47-52]. Apoptosis is a regulated process modulated by both pro-apoptotic and anti-apoptotic cellular factors and it can be considered an active process[53], while necrosis is the passive death of cells[54]. In any case, APCs death could also cause a failure in the onset of an efficient immune response, owing to cells could not properly activate T cells.

WHAT DO WE KNOW ABOUT THE INTERACTION BETWEEN PRRSV AND APCS?

PRRSV and the expression of active (MHC-Ⅱ) and co-stimulatory (CD80/86) molecules

Due to the complexity in the isolation and culture of DCs from different porcine organs[55,56], the vast majority of conducted studies are *in vitro* studies.

After the infection of monocyte-derived dendritic cells (MoDCs) with either PRRSV-1 or PRRSV-2 strains, the expression of MHC-Ⅱ decreased[32,34,35] or remained unaltered[37]. The expression of MHC-Ⅱ in BMDCs infected with a PRRSV-2 strain did not show any change in its expression[33,36]. Nevertheless, according to Gimeno *et al*[5] in which 4 selected PRRSV-1 strains were used (one IL-10$^+$-TNF-α$^+$ strain, one IL-10$^-$-TNF-α$^+$ strain, one IL-10$^+$-TNF-α$^-$ strain and one IL-10$^-$-TNF-α$^-$ strain), infected-BMDCs exhibited either an increased expression of MHC-Ⅱ or no changes. Three out of four of these strains induced high expression of SLA-Ⅱ, while the IL-10$^-$-TNF-α$^+$-prototype strain did not evidence any change. Therefore, the use of different genotypes on different or the same subpopulation of APCs leads to different outcomes[5].

With regard to the expression of CD80/86 molecules, some authors pointed out a decrease in the expression of these molecules on MoDCs[34], while others mentioned an increased expression on these cells[35]. With regard to BMDCs, a decrease[5], no changes[5] and an increased expression of CD80/86[5,33,36] have been reported. Interestingly, in the article published by Peng *et al*[36], it was observed that both, bystander and PRRSV-infected cells, showed high expression of CD80/86 which may be associated with the release of soluble factors by infected cells or the engulfment of infected and/or apoptotic DCs. In fact, in the above mention study from Gimeno *et al*[5], the IL-10$^-$-TNF-α$^-$ prototype strain leads to the highest increase in the expression of CD80/86 in BMDCs while the double positive one, induced a decrease in CD80/86 compared to mock-infected group. It demonstrates that the behaviour of each strain can vary depending on the induced-cytokine profile.

The diminished expression of MHC-Ⅱ has also been linked to a lack of proliferation of leucocytes when co-

cultured with PRRSV-infected DCs, suggesting that PRRSV might modulate the immune stimulatory function of porcine DCs[35]. Moreover, in most of the above mentioned studies, only one of the two molecules (either MHC-Ⅱ or CD80/86) was increased and both of them are mandatory for the correct activation of T cells[26,27]. These findings highlight the complexity of the immune response against PRRSV, which may be triggering off different mechanisms to evade the host immune response not only in PRRSV-infected cells but also in bystander non-infected cells.

The *in vivo* expression of MHC-Ⅱ, as well as, CD80/86 on APCs has been poorly studied. In a study carried out by our research group, pigs which had been infected with a PRRSV-1 strain showed a decrease in the number of macrophages, as well as, the expression of MHC-Ⅱ in the tonsil, retropharyngeal and mediastinal lymph nodes compared to uninfected pigs[43]. In addition, a significant negative correlation was found between the expression of PRRSV antigen and the number of human leucocyte antigen-DR (HLA-DR) positive cells. Studying consecutive immunohistochemical sections, we observed that most of PRRSV antigen-positive cells were negative for HLA-DR antigen[43], pointing out a downregulation of MHC-Ⅱ in PRRSV infected cells.

PRRSV and cell death

Not only PRRSV could alter the expression of molecules involved in antigen presentation. Other way to abrupt antigen presentation is causing the death of APCs. Thus, concerning this point, several *in vitro* and *in vivo* studies have been conducted.

Concerning *in vitro* experiments, some authors observed apoptosis in bystander non-infected cells of American Type Culture Collection CRL11171 cell line[49] at the same time that other authors perceived co-localization of both, apoptotic and PRRSV antigens on macrophages and MARC-145 cells[57]. According to these authors, PRRSV is first able to provoke an anti-apoptotic state on cells suffering viral replication, inducing apoptosis later when the replication cycle has taken place. However, not only death has been observed during PRRSV infection, but also necrosis of MARC-145 cells[58,59].

Regarding MoDCs and BMDCs, cell death after PRRSV infection has been poorly studied. Both, apoptosis[32,34] and necrosis[32] phenomena have been noticed, although no co-localization of apoptotic or necrotic markers with PRRSV antigen were studied.

In vivo studies have evidenced apoptotic cells in testis[48], lungs and lymphoid organs of PRRSV-1 and PRRSV-2 infected pigs[49-52]. Although apoptosis has been associated with GP5 of PRRSV in infected cells[47,60], cell death has also been reported in non-infected bystander cells[49,50,52]. However, no co-localization of apoptotic markers and PRRSV expression has been analysed. This approach suggests that besides a direct induction of apoptosis by viral particles, an indirect pathway of apoptosis play a role in cell death during PRRSV infection. Several attempts have been carried out to relate indirect apoptosis of PRRS to the release of some apoptogenic cytokines, such as, TNF-α[61], IL-1 or IL-10[51]. Nonetheless, some of these associations could not be confirmed by *in vitro* studies with recombinant porcine cytokines[51]. Other studies have shown an enhanced expression of both Fas and FasLigand in PRRSV-2 infected splenic macrophages and in co-cultured splenic and peripheral blood lymphocytes[62], highlighting the necessity of exploring the role of different apoptotic mediators in PRRSV-induced cell death.

FUTURE ASPECTS AND ADVICES TO HEED IN THIS ISSUE

The expression of MHC-Ⅱ and CD80/86 has been analysed in different *in vitro* DC-models. However, these studies lack of the co-localization of PRRSV and the molecule involved in. Moreover, strains with different profile of cytokine release lead to different results. Therefore, co-localization studies, as well as, cytokine analyses should be performed in order to obtain clearer results on PRRSV modulation of the host immune response. Key cytokines might be interferon (IFN)-α and IFN-γ, because of their antiviral properties; TNF-α, due to anti-inflammatory, antiviral and apoptogenic functions; and IL-10, because of its immunomodulatory and apoptogenic properties. By doing so, it will be clarified if the virus itself, different cytokines, or both of them are able to cause a change in the expression of these molecules.

As above mentioned, TCR-MHC-Ⅱ and CD28-CD80/86 signals are mandatory to properly activate T cells. Thus, it is necessary to study both molecules in every conducted experiment to extrapolate and ensure the behaviour of these molecules.

A decreased expression of MHC-Ⅱ, CD80/86 or both of them could result in a failure or, at least, a non-effective immune response. In an *in vitro* study carried out in our group (data not published), it has been observed an enhanced expression of both molecules, MHC-Ⅱ and CD80/86, in MoDCs infected with a PRRSV-1 strain which had previously been tested for inducing a strong activation of the immune response. However, no proliferation of T cells was observed in this study and, on the contrary, a high rate of dead cells was detected. Therefore, a new strategy of the virus could be drawn, by which, although the virus induces the expression of MHC-Ⅱ and CD80/86 in MoDCs, they result ineffective since the virus later on induce their cell death. Thus, the use of cell-death markers should be also included in our routine experiments.

The same view should be extrapolated to death pathways studies. Moreover, future foresight experiments should broaden the spectrum of APC types and PRRSV strains in order to generate a clearer picture of this disease. The consideration of these aspects will improve the current knowledge on the pathogenesis and immune response against this virus, paving the way for its control.

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BRIEF ARTICLE

Genetic analysis of structural proteins in the adsorption apparatus of bacteriophage epsilon 15

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Author contributions: McConnell MR designed and performed the genetic mapping and DNA sequencing experiments and also directed undergraduate researchers; Guichard JA and Middleton PC, in performing compositional analyses of particles produced by nonsense mutant viruses and the wt parent strain under non-permissive growth conditions; McConnell MR interpreted the resulting data and wrote the manuscript.

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Abstract

AIM: To probe the organizational structure of the adsorption apparatus of bacteriophage epsilon 15 (E15) using genetic and biochemical methodology

METHODS: Hydroxylamine was used to create nonsense mutants of bacteriophage E15. The mutants were then screened for defects in their adsorption apparatus proteins, initially by measuring the concentrations of free tail spike proteins in lysates of cells that had been infected by the phage mutants under non-permissive growth conditions. Phage strains whose infected cell lysates contained above-average levels of free tail spike protein under non-permissive growth conditions were assumed to contain nonsense mutations in genes coding for adsorption apparatus proteins. These mutants were characterized by classical genetic mapping methods as well as automated sequencing of several of their genes. Finally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were used to examine the protein compositions of the radioactive particles produced when the various mutants were grown on a non-permissive host cell in the presence of ^{35}S-methionine and co-purified along with E15wt phage on CsCl block gradients.

RESULTS: Our results are consistent with gp4 forming the portal ring structure of E15. In addition, they show that proteins gp15 and gp17 likely comprise the central tube portion of the E15 adsorption apparatus, with gp17 being more distally positioned than gp15 and dependent upon both gp15 and gp16 for its attachment. Finally, our data indicates that tail spike proteins comprised of gp20 can assemble onto nascent virions that contain gp7, gp10, gp4 and packaged DNA, but which lack both gp15 and gp17, thereby forming particles that are of sufficient stability to survive CsCl buoyant density centrifugation.

CONCLUSION: The portal ring (gp4) of E15 is bound to tail spikes (gp20) and the tail tube (gp15 and gp17); gp17's attachment requires both gp15 and gp16.

Key words: Epsilon15; Virion structure; Salmonella phages

Core tip: Epsilon 15 (E15) is a temperate, serotype-converting bacteriophage that specifically infects group E1 Salmonellae bacteria. This paper presents genetic and biochemical evidence regarding the identities and positional relationships of the proteins that comprise the tail tube structure of E15. As such, it makes a small contribution towards what may someday be a fuller understanding, not only of how E15 stabilizes its packaged DNA, but also, how it triggers release of its DNA

when the phage encounters a susceptible Salmonella host cell.

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INTRODUCTION

Salmonella bacteria are enteric organisms that constitute a serious source of gastro-intestinal infection in humans and agriculturally important animals[1]. Bacteriophages provide an important mechanism of genetic variation and gene exchange among Salmonella bacteria (and thus, the potential for enhanced pathogenicity) through their ability to promote lateral transfer of host cell genes. Understanding the structural features of phage DNA packaging and adsorption/DNA ejection apparati is an important step in being able to fully assess how phage contribute to genetic variation within their Salmonella hosts.

Bacteriophage epsilon15 (E15) is a temperate, Group E1 Salmonella-specific phage that belongs to the Order "Caudovirales" and the Family "Podoviridae"[2]. At the genomic level[3], it closest relatives are the Salmonella-specific viruses, SPN1S (NCBI Accession number JN391180.1) and SPN9TCW (NCBI Accession number JQ691610.1) but it also shares 36 related genes in common with the *E. coli* O1H57-specific phage, ϕV10 (NCBI Accession number DQ126339.2). E15 was among the first Salmonella-specific phages to be discovered and was a popular experimental model for Japanese and US investigators in the 50's, 60's and 70's, both because of its ability to cause serotype conversion and because of its enzymatically active tail spikes, which display endorhamnosidase activity towards the host cell O-polysaccharide structure[4-9]. The publication of the E15 genome sequence by our laboratory in 2002 (NCBI Accession number AY150271.1) stimulated renewed interest in E15, this time as a model system for investigating virion structure by cryo-electron microscopy (cryo-EM), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and other methods[3,10-14]. These studies, combined with earlier genetic and biochemical investigations[6], have revealed the following: (1) gp7 and gp10 together comprise the capsid of E15; (2) E15's enzymatically active tail spikes are homotrimers of gp20; and (3) other major proteins in E15 virions include gp4, gp15 and gp17. Circumstantial evidence, including size, relative abundance within virion particles and the position of its gene just downstream of those coding for the small and large terminase subunits in the late transcript are all consistent with gp4 being the portal protein of E15[3].

In addition to being a powerful tool for elucidating virion capsid structures, cryo-EM can also be used effectively to decipher the structure of a phage adsorption apparatus, especially if the adsorption apparatus can be detached intact from the virion capsid and prepared in purified form. Such was the case for the Group B Salmonella-specific phage, P22, and the resulting structure that was determined by cryo-EM analysis of these P22 adsorption apparati (termed "tail machines") is, in a word, spectacular[15,16]. To date, no one has reported having successfully purified the intact adsorption apparatus of phage E15.

In this paper, we present genetic and biochemical data that is consistent with gp4 forming the portal ring structure of E15; in addition, our data indicates that the centrally-positioned tail tube portion of the adsorption apparatus is likely comprised of gp15 and gp17, with gp17 being more distally positioned than gp15 and dependent upon both gp15and gp16 for its attachment. Finally, our data indicates that tail spike proteins comprised of gp20 can form stable associations with nascent virus particles that contain gp7, gp10, gp4 and packaged dsDNA, but which lack both gp15 and gp17. This implies that tail spikes bind directly to the portal ring during the assembly process that leads to the formation of mature virions.

MATERIALS AND METHODS

Phage and bacterial strains

Parental phages E15 and E15vir (a clear plaque mutant with a missense mutation in gp38, the major repressor protein) as well as bacterial host strains *Salmonella enterica subsp. enterica* serovar Anatum A1 *and Salmonella enterica subsp. enterica* serovar Anatum 37A2Su+ all came originally from the laboratory of Dr. Andrew Wright (Tufts University, Boston, MA). E15 (am2) is a nonsense mutant of E15 that is unable to produce tail spike proteins[6]. Propagation of bacteria and phage was in trypticase soy broth, unless otherwise indicated.

Isolation of phage nonsense mutants with adsorption apparatus defects

Nonsense mutants of E15vir were generated by hydroxylamine mutagenesis[17] and were detected initially by an anaerobic, double layer plating method that dramatically increases plaque size[18]. Hydroxylamine-treated phage were mixed with an amber suppressor strain (*Salmonella anatum* 37A2Su+) in the bottom LB soft agar layer, then overlaid with a second soft agar layer containing the non-suppressing parental strain *Salmonella anatum* A1. Turbid-looking plaques were cloned and re-screened to verify their inability to form plaques on *Salmonella anatum* A1.

Phage nonsense mutants isolated by the method described above were subsequently screened individually for potential defects in adsorption apparatus proteins other than the tail spike by measuring the level of free tail spike protein in lysates of non-permissively infected cells. The tail spike assay was based on a method developed earlier in an investigation involving phage P22 tailspikes[19]; It involved UV-irradiating 10000RPM (10K) supernatant frac-

tions obtained from lysates of *Salmonella anatum* A1 cells infected by E15vir nonsense mutants, then incubating the irradiated 10K supernatants with E15 "heads" obtained by infecting *Salmonella anatum* A1 with E15 (am2), an E15 nonsense mutant that is unable to produce tail spike protein. Following incubation, reaction mixes were plated at varying dilutions on the permissive host strain, *Salmonella anatum* 37A2Su+, in order to titer the number of E15 (am2) "heads" that were made infectious by the binding of tail spike proteins *in vitro*.

Genetic mapping and sequencing of Epsilon15 nonsense mutations: E15vir nonsense mutants isolated and screened as described above were characterized (along with the known tailspike nonsense mutant, am2) using classical *in vivo* complementation and two-factor recombination assay procedures that have been previously described[6]. These genetic mapping studies revealed the number of complementation groups (*i.e.,* genes) defined by the nonsense mutants and also allowed for an approximation of their locations relative to the E15 tail spike gene. Shortly after the mapping of the nonsense mutations using classical methods, the genomic sequence of E15 was completed by our lab. Gene 20 was then shown by sequencing analysis to contain the am2 nonsense mutation (*i.e.,* gp20 is the tailspike protein) and in addition, was observed to be the distal-most gene in the late mRNA transcript of E15[3].

Each E15vir mutant believed to be defective in an adsorption apparatus protein was subjected to DNA sequence analyses for genes 15, 16 and 17, in an effort to assign a gene identity for its nonsense mutation. The bracketing, Frwrd and Rvrse primer pairs used for initial PCR amplification of the three genes are shown below, with underlined bases representing modifications made in order to facilitate cloning of the PCR products into plasmids. Gene 15: *E15.Orf15*.Frwrd, AGGGATCCAAATGCCAGTTGTACCTACAG, *E15.Orf15*.Rvrse, ATACATAAGCTTTTATTCAACCCTCACG; Gene 16: *E15.Orf16*.Frwrd, TGGATCCATGGCTGATGTATTTTCACT, *E15.Orf16*.Rvrse, ACACATGCCTGCAGCATTATGGATTCCT; Gene 17*: E15.Orf17*.Frwrd, GAGGGATCCATAATGAAACAGGCATGTGT, *E15.Orf17*.Rvrse, GTTAAGGGTACCATCATTGTCCTA.

Because of their large sizes (ranging from 1928 to 2782 basepairs), the resulting PCR products were sequenced not only with the same Frwrd and Rvrse primers that had been used to produce them, but also with several additional primers known to bind internally within each PCR product. The internal sequencing primers were as follows: Gene 15: *E15.g15.W12689*: GGCGCTGCTCATGGCTGGAGTCATGAACAG, *E15.g15.W13264*: CGCGGCTATCGGTCTTTCTCAGTTACCTAC, *E15g15.W13879*: GGAGGCGGCTGCGCTGTCTGAACAGGTAC; Gene 16: *E15.g16.W15213*: CGGCAGGCATGGCCCTTCCTGCTGCTGTTG, *E15.g16:W15689*:TAGCGAACAGCCAGCGCATCCTGGATAAC; Gene 17: *E15.g17.W17092*: GCGGCAAAGTCTGCACAGTTCCAGATCCTG, *E15.g17.W17717*: GACCTGACGCTGCGCGAAACTTTTCCCTTG, *E15.g17.W18214*: GCGGCGTTCGGGCTGTTGATGTACAAAAAC.

Taq polymerase is somewhat error-prone[20], so in order to generate PCR products suitable for accurate DNA sequencing, PCR reaction mixes were prepared on a large scale (250 μL), then separated into five 50 μL aliquots prior to commencing the thermocycling reaction. Upon completion of PCR, the five aliquots were recombined into a single 250 μL sample and the DNA product was purified using a QIAGEN PCR purification column. Automated DNA sequencing reactions were performed by the Microchemical Core Facility at San Diego State University.

Preparation and analysis of ^{35}S-methionine labeled, virion-like particles produced by phage nonsense mutants under non-permissive conditions: Preparations of ^{35}S-methionine labeled, wild type E15vir phage particles and non-infectious, virion-like particles produced by the nonsense mutants were obtained by incubating mid-log phase *Salmonella anatum* A1 cells grown in low sulfate medium with phage (multiplicity of infection of 10) for ten minutes at 0 °C, then adding ^{35}S-methionine to a final concentration of 10 uCi/mL and shifting the incubation temperature to 37 ℃. At T = 90 min, cell cultures were lysed with chloroform, then centrifuged for 10 min at 10000 RPM in order to remove cellular debris. The resulting 10K supernatant fractions were loaded onto CsCl block gradients and centrifuged for 30 min at 38000 RPM on a Beckman L8-80M ultracentrifuge (an excess of cold E15wt phage was included in each sample as a carrier). Particles displaying virion-like densities (*i.e.,* the ability to pass readily through a 1.375 g/cm^3 CsCl layer and settle onto a 1.6 g/cm^3 CsCl layer along with non-radioactive E15wt carrier phage) were dialyzed, normalized for cpm and electrophoresed on 12% sodium dodecyl sulfate-protective antigen (SDS-PA) gels. The gels were subsequently dried on Whatman 3M paper and the paper was exposed to Kodak X-Omat X-ray film in order to detect radioactive proteins by autoradiography.

RESULTS

Isolation and mapping of E15 nonsense mutants with adsorption apparatus defects

We reasoned that cell lysates produced by infection of *Salmonella anatum* A1 with E15vir phage containing nonsense mutations in genes coding for adsorption apparatus proteins other than the tail spike should contain higher than normal levels of free tail spike protein. Cell lysates produced by infection with different E15 nonsense mutants were therefore screened for their ability to provide tail spike proteins to E15 (am2) "heads" *in vitro*, thereby rendering the heads infectious. Six E15vir nonsense mutants whose lysates had tail spike levels surpassing that

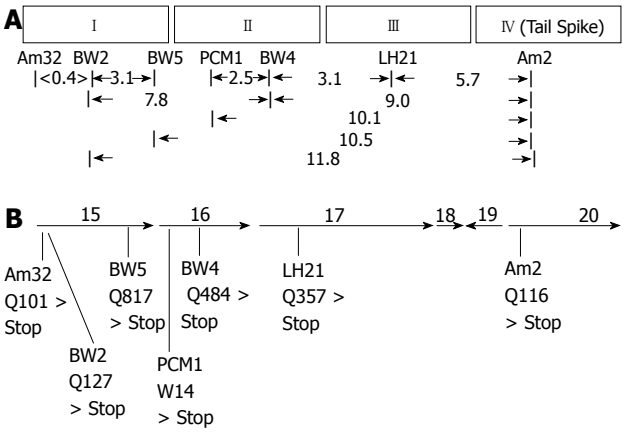

Figure 1 Genetic mapping and sequencing data showing positions of nonsense mutations that affect the protein composition of the epsilon 15 adsorption apparatus. A: Two-factor recombination values for nonsense mutations falling within *in vivo* complementation groups I through IV; B: Gene sequencing data. PCM1: Pericentriolar material 1; LH: Luteinizing hormone.

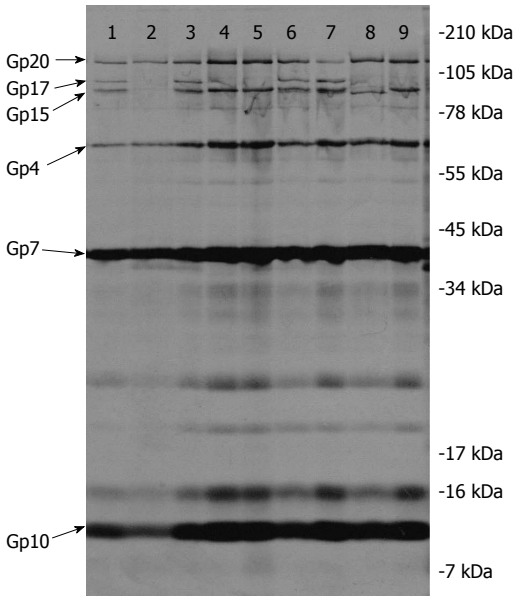

Figure 2 Autoradiogram showing compositions of non-infectious epsilon 15Vir particles. Lanes 1, 3 and 6, E15vir; Lane 2, gene 15 mutant am32 (BW2 is not shown but gives an identical pattern); Lanes 4 and 5, gene 16 mutants pericentriolar material 1 and BW4; Lane 7, partially suppressed am2 (gp20-) particles; Lane 8, gene 15 mutant BW5; Lane 9, gene 17 mutant luteinizing hormone21. molecular weight markers are depicted to the right.

of an E15vir lysate were identified, then further analyzed using classical genetic mapping methods. The six mutants were shown to define three complementation groups (*i.e.,* genes), which mapped in close proximity to each other as well as to the tail spike gene, defined by nonsense mutation am2 (Figure 1A). After confirming by DNA sequencing that the am2 mutation lay within gene 20 (the last gene in E15's "late" mRNA transcript), PCR primers were used to amplify and sequence three genes for each of the six mutants; namely 15, 16 and 17. Genes 15 and 17 were chosen for sequence analysis because the pI values, overall sizes, and tryptic digestion fragment sizes of their inferred polypeptide products closely matched those of E15 virion proteins shown by SDS-PA/autoradiography to be missing in virion-like particles formed by the various nonsense mutants under non-permissive conditions[3]. Gene 16 was included for sequence analysis as well because the genetic mapping data showed that the collection of six nonsense mutations with potential adsorption apparatus defects defined three different genes. Other neighboring genes (*i.e.,* 13, 14, 18 and 19) all coded for inferred proteins that were either very small or strongly hydrophobic, and were therefore not included in the sequencing analysis.

The DNA sequencing data (Figure 1B) revealed the presence of unique amber nonsense mutations in gene 15 for the three non-complementing phage mutants am32, BW2 and BW5. Non-complementing mutants pericentriolar material 1 (PCM1) and BW4 both contained unique amber nonsense mutations in gene 16, while mutant luteinizing hormone 21 (LH21), which the classical mapping data showed to be in a complementation group of its own, was found to contain a unique amber nonsense mutation in gene 17. The positions of the nonsense mutations determined by DNA sequencing correlated nicely with the linear map order that had been established for them previously by recombination analysis. In every case, the nonsense mutation had resulted from a hydroxylamine-induced C > T transition (either CAG > TAG, or TGG > TAG).

Yields and polypeptide compositions of E15 nonsense mutants with adsorption apparatus defects

MALDI-TOF mass spectrometry analyses of trypsin-digestion products obtained from purified E15 virion proteins[10] indicate that after the tail spike protein, gp20 (1070 amino acids, 115676 daltons), the next two largest proteins contained in E15 virions are gp17 (918 amino acids, 100841 daltons) and gp15 (842 amino acids, 91012 daltons). When ^{35}S-methionine-labeled particles produced by the various nonsense mutants under non-permissive conditions were co-purified with non-radioactive, "carrier" E15wt phage on CsCl block gradients, then analyzed by SDS-PAGE and autoradiography, it was observed that the two gene 16 mutants (PCM1 and BW4) and the gene 17 mutant (LH21) all produced good yields of radioactive particles relative to E15wt (118%, 154% and 100%, respectively, with a mean of 124 ± 28% SD) and that these particles all lacked gp17 (Figure 2, Lanes 4, 5 and 9). The three gene 15 mutants (am32, BW2 and BW5) all produced lower quantities of radioactive particles than E15wt (17%, 23% and 44%, respectively, with a mean of 28 ± 14% SD). The am32 and BW2 mutants, whose nonsense mutations mapped at codons 101 and 127, respectively, of gene 15 (845 codons), produced particles that lacked both gp15 and gp17 (Figure 2, Lane 2). Mutant BW5, whose nonsense mutation maps at codon 817 of gene 15, produced particles lacking gp17 but containing a novel protein with a slightly faster mobility than that of gp15; a protein most

likely comprised of amino acids 1 through 816 of gp15 (Figure 2, Lane 8). The quantity of the slightly truncated gp15 protein in BW5 particles is reduced, relative to the quantity of gp15 observed in E15vir and the various gp17-deficient mutants (see Lane 9, for example), thus indicating that its ability to assemble onto nascent virion particles has been diminished by the loss of 29 C-terminal amino acids, but not entirely eliminated. The 10K supernatant fractions obtained from cells infected by the three gene 15 mutants (am32, BW2 and BW5) were also analyzed by SDS-PAGE and autoradiography. All three supernatants contained a protein that co-migrated with the gp17 protein of E15wt (data not shown).

The two gene 16 nonsense mutants analyzed in this study (PCM1 and BW4) both produced good yields (118% and 154%, respectively, relative to wt E15) of non-infectious, virion-like particles that are missing gp17 (Figure 2, Lanes 4 and 5). As was the case for the three gene 15 mutants, a protein with gp17-like mobility was present in the 10K supernatant fractions of cells infected by PCM1 and BW4 (data not shown).

Every nonsense mutant that was studied produced radioactive particles that contained DNA, as judged by their ability to co-sediment with E15wt virions through CsCl at 1.375 g/mL and layer onto the 1.6 g/mL solution. In addition, all of the mutants, whether gp17-deficient or both gp15- and gp17-deficient, displayed normal quantities of the two known capsid proteins, gp7 and gp10, as well as gp4. Yields of the radioactive particles that lacked both gp15 and gp17 were significantly lower than those of particles that lacked gp17 only, suggesting that maximum stability of packaged DNA is achieved when both gp4 and gp15 are present. All of the mutant phage particles contained sufficient gp20 tail spike protein for easy detection by autoradiography (see lanes 2, 4, 5, 8, 9 of Figure 2).

DISCUSSION

The complete absence of both gp15 and gp17 in high-density particles produced by mutants am32 and BW2, whose nonsense mutations both map near the beginning of gene 15, combined with the gp17-only deficiency observed in high density particles produced by the gene 17 nonsense mutant (LH21), argues for a model in which gp15 and gp17 occupy penultimate and terminal positions, respectively, within a peripheral E15 virion structure that we hypothesize is the tail tube. The missing 29 amino acids at the C-terminal end of the gp15-like protein that is produced by BW5 phage under non-permissive conditions must be critical for gp17 binding since no gp17 protein was detected in these particles.

We currently do not know why gp16 is required for gp17's assembly onto nascent virions. The gp16 protein is inferred to have 634 amino acids and our two gene 16 nonsense mutations, PCM1 and BW4, are positioned at codons 14 and 484, respectively. The predicted mass for gp16 is 67364 daltons and its inferred overall methionine content (2.4%) falls within the range of methionine contents inferred for the other known virion proteins (from as low as 1.3% for gp20 to as high as 5.2% for gp4). In other words, if gp16 is present in E15 virions in appreciable quantities, then it should contain sufficient ^{35}S-methionine to show up in our autoradiogram. Faint protein bands were observed above the 78 kDa marker and above and below the 55 kDa marker on the gel (Figure 2), but none of these three proteins appeared to be diminished in quantity in the gene 16 mutants, relative to the other mutants or to E15vir. It is conceivable that gp16 is a virion protein that was not detected in our experiment because it co-migrated with gp4 protein (the inferred mass for gp4 is 61657 daltons). If that is true, though, one can argue that the quantity of gp16 in virions must be quite small, since the intensities of the gp4 bands in the two gene 16 mutants do not appear to be diminished, relative to those of E15vir and the other nonsense mutants that were analyzed. It should be noted that both our lab and at least one other have detected gp16 tryptic fragments in purified E15 virions using MALDI-TOF analysis[10]; the other lab has more recently hypothesized that gp16 is a tail tube protein[21]. While the data in this paper does not support that hypothesis, we remain open to the possibility and are continuing to explore the role played by gp16 in E15 virion assembly. It has also been hypothesized that gp17 functions as a pilot (or ejection) protein for E15[21]; this seems highly unlikely since ejection proteins, as the name implies, exit the capsid along with the DNA during the infection process[22,23]. Our results clearly show that E15 particles lacking gp17 retain stably packaged DNA within their capsids, as evidenced by their ability to co-purify in high yields with E15wt carrier phage on CsCl block gradients; furthermore, the same holds true, albeit to a lesser degree, for particles that are lacking both gp15 and gp17.

Frankly, we were surprised that tail spikes were present in all of the particles produced by our nonsense mutants. The initial screening procedure used to identify nonsense mutants for this study was based on the assumption that mutations resulting in adsorption apparatus defects would hinder tail spike assembly onto the virion, thereby resulting in higher than normal levels of free tail spike protein in the infected cell lysates, as well as the production of phage particles lacking tail spike proteins. Our current explanation is that gp4 forms the portal ring structure and perhaps, with help from immediately adjacent capsid proteins, provides a significant part of the binding surface(s) to which gp20 tail spikes normally attach during virion assembly. Interestingly, in their first cryo-EM paper dealing with E15, Jiang *et al*[10] reported that two of E15's six tail spikes occupy positions around the tail tube that place them in very close contact with the capsid. If these two tailspikes are more firmly bound in gp17- and gp15-/gp17-deficient particles than the other four, then that might explain both the presence of gp20 in the mutant particles as well as the enhanced levels of tail spike protein in their infected cell lysates.

Figure 3 sums up our current model for the structure of the E15 adsorption apparatus: (1) gp4 forms the

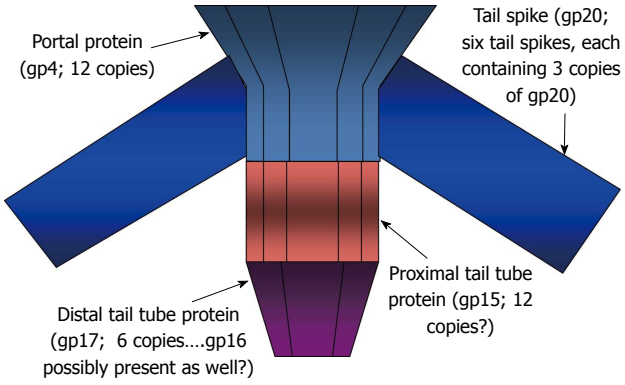

Figure 3 Schematic model for protein positions and interactions within the adsorption apparatus of bacteriophage Epsilon 15. The estimates of 12 and 6 copies for gp15 and gp17, respectively, are based upon stoichiometric measurements made relative to the numbers of capsid and tail spike proteins present in epsilon 15[13]; tail spike attachment to portal protein may be further stabilized by interactions with gp15 and/or capsid proteins.

portal ring structure and perhaps, with help from neighboring capsid proteins, provides a binding surface that is sufficient for attachment of tail spikes (gp20); (2) gp15 and gp17 form the central tail tube, with gp17 occupying the more distal position and interacting with gp15 by 4° interactions that cannot occur if the C-terminal 29 amino acids of gp15 are missing. The association of gp17 with gp15 is also gp16-dependent but we do not know yet whether or not gp16 forms part of the tail tube. We are currently continuing our study of E15 adsorption apparatus structure and function by conducting phenotypic suppression experiments with an E15 mutant in our collection that under non-permissive conditions, adsorbs to cells and degrades O-polysaccharide normally, but fails to eject its DNA[6].

The best understood Salmonella-specific phage in the Podoviridae family is P22 and recent X-ray crystallography and cryo-EM studies have revealed features of the proteins that comprise its capsid, portal, tail tube, needle and tail spikes in exquisite detail[15,16,24,25]. The dodecameric, ring-shaped portal structure of P22 is comprised of gp1; below the portal ring is the tail tube, comprised of twelve copies of gp4 (bound directly to the portal) and six copies of gp10, which are bound to gp4. Attached to the distal portion of gp10 is P22's "needle" structure, which is comprised of three copies of gp26. The six laterally-positioned, homo-trimeric tail spikes of P22 are comprised of gp9 and are thought to be associated with a binding surface generated cooperatively by proteins gp4 and gp10 at their point of junction on the sides of the tail tube[15].

Gene homology studies indicate that of the three Podoviridae phages known to infect Group E Salmonellae, namely E15, Epsilon34 (E34) and g341, two (E34 and g341) likely have adsorption apparatus protein compositions and organizations that are similar to that of P22[26,27]. Phage E15, on the other hand, has clearly taken a different path; Its tail spike protein is gp20, which at 1070 amino acids (aa) is about 63% larger, on average, than those of E34 (606 aa), g341 (705 aa) and P22 (667 aa) and is homologous with them only in a short stretch of amino acids at the N-terminal end that are thought to be critical for assembly onto the virion. Although they appear to occupy similar positions in the tail tube, there is no apparent structural homology between the proximal tail tube proteins of E15 and P22 (gp15 and gp4, respectively) or between their distal tail tube proteins (gp17 and gp10, respectively). There are stoichiometric similarities, though, in that densitometry measurements of Coomassie Blue-stained proteins of wild type E15 virions, followed by normalization for size differences, indicate that tail spikes (gp20), proximal tail tube proteins (gp15) and distal tail tube proteins (gp17) are present in E15 virions at approximately a 3/2/1 ratio, which matches the well-established 18/12/6 ratios of tail spike (gp9), proximal tail tube (gp4) and distal tail tube (gp10) proteins known to be present in P22 virions. No homolog of the P22 "needle" protein (gp26) is present among inferred bacteriophage E15 proteins, but that is not surprising since the tail tubes of negatively-stained E15 virions do not display the "needle-like" protuberance that is seen in electron micrographs of P22[6]. The "needle" is thought to play a role in the movement of the P22's genome across the bacterial cell envelope during an infection[28]. How E15 compensates for its lack of a "needle" protein remains to be determined.

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COMMENTS

Background

In April, 2010 (http://www.hhs.gov/asl/testify/2010/04/t20100428b.html), the Director of the Center for Disease Control reported to the House Subcommittee on Human Health that approximately 1.4 million Americans are infected annually with foodborne strains of Salmonellae bacteria. He further stated that the incidence of antibiotic resistance among isolates of Salmonella strains obtained in hospitals, stock animals and the food supply were all on the rise. Generalized transduction by bacteriophages is a major method for the horizontal transfer of genes between Salmonella organisms and thus, likely plays a role in their evolving pathogenicity.

Research frontiers

The ability of a bacteriophage to infect a bacterium is governed by the nature of its adsorption apparatus. The adsorption apparatus is a collection of proteins that cooperate together to maintain the stability of the phage's packaged genome until the moment when a susceptible host cell is encountered. At that point, the same sets of proteins interact with each other in an entirely different manner to trigger ejection of the phage genome and facilitation of its transport into the host cell cytoplasm.

Innovations and breakthroughs

Recent cryo-electron microscopy studies on virions of the Group E1 Salmo-

nella-specific bacteriophage, epsilon 15 (E15) have yielded highly detailed information on the composition and structure of the phage's capsid. Those same investigators have also produced the first close-up view of the adsorption apparatus of E15. This paper presents data regarding the identities of the proteins that comprise E15's adsorption apparatus; in addition, the data presented herein provides some insight into the ways these proteins interact with each other in order to form the adsorption apparatus.

Applications

Compared with other salmonellae-specific members of the podoviridae family, bacteriophage E15 appears to be unique when it comes to the collection of proteins that comprise its adsorption apparatus. Perhaps, in addition to the uniqueness of their physical characteristics, the manner in which these proteins interact with each other to control the stability of packaged DNA as well as its release in response to the proper environmental cue will also prove to be novel, and thus, worthy of further study.

Terminology

Adsorption apparatus pertains to those proteins that are stably associated with the mature virion, either through direct binding interactions with the portal ring or else, by virtue of their association with other proteins that are bound to the portal ring.

Peer review

The authors used genetic and biochemical methods to examine compositional and organizational aspects of the adsorption apparatus of bacteriophage E15. Although preliminary, the results are sufficient for establishing a simple model that should be possible to refine with further experimentation.

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BRIEF ARTICLE

Evolution of an avian H5N1 influenza A virus escape mutant

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Abstract

AIM: To investigate the genetic constitution of an escape mutant H5N1 strain and to screen the presence of possible amino acid signatures that could differentiate it from other Egyptian H5N1 strains.

METHODS: Phylogenetic, evolutionary patterns and amino acid signatures of the genes of an escape mutant H5N1 influenza A virus isolated in Egypt on 2009 were analyzed using direct sequencing and multi-sequence alignments.

RESULTS: All the genes of the escape mutant H5N1 strain showed a genetic pattern potentially related to Eurasian lineages. Evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the escape mutant strain while confirming close relatedness to other H5N1 Egyptian strains from human and avian species. A variety of amino acid substitutions were recorded in different proteins compared to the available Egyptian H5N1 strains. The strain displayed amino acid substitutions in different viral alleles similar to other Egyptian H5N1 strains without showing amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

CONCLUSION: The genetic characteristics of avian H5N1 in Egypt revealed evidence of a high possibility of inter-species transmission. No amino acid signatures were found to differentiate the escape mutant H5N1 strain from other Egyptian H5N1 strains.

Key words: Chicken; Genotyping; H5N1; Influenza; Virus evolution

Core tip: The evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the examined escape mutant H5N1 strain; however, a variety of amino acid substitutions were recorded. The displayed amino acids substitutions in different viral alleles denoted considerable possibility of inter-species transmission, virulence to mammalian species and cytokine resistance.

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INTRODUCTION

The influenza A viruses belong to the Family Orthomyxoviridae. The hemagglutinin (*HA*) and neuraminidase (*NA*) genes encode viral envelope proteins and there are 17 *HA* and 10 *NA* subtypes[1]. Other influenza genes include *PB2*, *PB1*, *PA*, *NS*, *M* and *NP* that encode for viral internal proteins, are required for viral replication and assembly[2] and play an important role in viral infectivity[3]. Reassortments between different influenza A subtypes H9N2 and H5N1 or H7N3 have been detected[4,5]. Interspecies transmission can lead to catastrophic consequences. Egyptian H5N1 viruses are classified as clade

Table 1 Oligonucleotides used for amplification of the *H5N1* genes

Locus	Name	Primer sequence	Length	Amplicon size (bp)	Location	Ref.
N1	N1-F	ATGAATCCAAATCAGAAG	18	1350	21-38	[38]
	N1-R	TGTCAATGGTGAATGGCAAC	20		1346-1365	
PB2	B2-F	GAGGCGATCTGAATTTCG	18	986	1256-1273	[39]
	B2-R	TATGCTAGAGTCCCGTTTCC	20		2222-2241	
PB1	B1F	AGCGAGGAGTATCTGTGAGA	20	601	774-793	[40]
	B1R	TTCCCTCATGATTCGGTGCA	20		1356-1375	
PA	PA-F	ATGAAGAGAGCAGGGCAAGA	20	868	491-510	This study
	PA-R	CAATGGGATACTTCCGCTGT	20		1339-1358	
NP	NP-F	TGCTTGCCTGCTTGTGTGTA	20	665	823-842	[39]
	NP-R	TACTCCTCTGCATTGTCTCCGA	22		1466-1487	
M	M-F	CCCTCAAAGCCGAAATCGCGCA	22	875	56-77	[40]
	M-R	TGCTGTTCCTGCCGATACTCTTCCC	25		906-930	
NS	NS-F	CACTGTGTCAAGCTTTCAGG	20	798	23-42	[39]
	NS-R	TCTCTTGCTCCACTTCAAGC	20		786-805	

2.2.1, which is further subdivided into two groups: A (A1-A5) and B (B1-B2)[6]. The economic consequences, in addition to the zoonotic implications, of highly pathogenic avian influenza virus H5N1 continue to constitute an important problem. According to the recent report of the World Health Organization in June 2013, 628 H5N1 infected cases with 374 fatal consequences were recorded. Egypt is among the countries that contain a very high number of the infected human cases (172) with a total of 62 fatal cases[7]. Endemic situations of H5N1 in Egypt is still an unsolved problem[8]. In Egypt, vaccination of poultry with inactivated vaccine preparations is currently adopted to combat H5N1; however, vaccination of household poultry was suspended in mid 2009 due to the limited impact on H5N1 incidence[8]. In turn, so-called "escape mutants" resulting from antigenic drift of the viruses are selected[9,10]. Escape mutants are known to be less liable to neutralizing antibodies induced by vaccines. Influenza viruses showed a considerable capacity to cross species barriers and to infect and be transmitted among susceptible mammals, including humans. Point mutations and allelic combinations possess a crucial effect on the virulence of HPAI H5N1 isolates and are thought to be polygenic[11,12]. Genetic reassortments among avian influenza viruses are commonly detected in wild bird and poultry isolates[13,14]. The possibility that an avian influenza virus, H5N1, can evolve to human-to-human or mammal-to-mammal transmission through the acquisition of genetic material from the other influenza viruses subtypes already circulating in human or mammals is not weak. The currently studied strain is an escape mutant strain that belongs to 2.2.1, B2 sublineage[10]. The current study aimed to investigate the genetic constitution of the escape mutant strain and compare it with other influenza strains. It also aimed to screen the presence of possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

MATERIALS AND METHODS

Viral RNA extraction and RT PCR

Viral RNA was extracted from the infective allantoic fluid of A/chicken/Egypt/F10/2009 using a spin column purification kit (Koma Biotech. Inc., South Korea). Amplification of viral genes was performed with gene-specific primers for *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* (Table 1) using a Koma one step RT PCR kit (Koma Biotech. Inc., South Korea). Following electrophoresis in a 1.5% agarose gel, bands of expected sizes were excised and purified using a QIAquick gel extraction kit (Qiagen, Germany). Purified amplicons were sequenced in both forward and reverse directions (Macrogen, South Korea). Sequences from different genes were routinely assembled and processed. Sequence data of the current study were submitted to the GenBank after removal of trimming primer-linker (Accession No. KC815941-KC815947).

Genetic and phylogenetic analysis

Sequence analysis of the viral genes was conducted using Mega 4.1 as previously described[15]. Sequence alignments of each of the seven genomic segments were conducted using the partial coding regions. Phylogenetic analyses of the A/chicken/Egypt/F10/2009 strain in the current study were conducted with other influenza A viruses to screen the possible reassortant allele. All gene sequence data were collected from the National Center for Biotechnology Information flu database. The neighbor-joining method with Kimura two-parameter distances was used for building the phylogenetic trees using the Mega 4.1[15]. The consistency of the internal branches was evaluated by the p-distance substitution model and 1000 bootstrap replications. The influenza A virus genotype tool at http://www.flugenome.org/genotyping.php[16] was used to determine individual genome segment lineages. A number of human, non-human mammalian and avian viruses were included in the evolutionary trees of *PB2, PB1, PA, NP, NA, M* and *NS* genes with selected sequences from different influenza serotypes in the GenBank to investigate relatedness and possible genetic reassortment.

Deduced amino acid sequence analysis

The multisequence alignment tool available in the flu database was used to compare the deduced amino acid

Table 2 Comparison of amino acid signatures in selected genes of avian and human strains to Egyptian H5N1 strains

Gene	Residue	Avian[1]	Human	Egyptian H5N1	A/CK/Egypt/F10/2009
PB2	475	$L^{214}M^{1}$	$M^{839}L^{3}$	$L^{52}M^{2}$	L
	588	$A^{203}/T^{6}/V^{6}$	$I^{835}/V^{3}/A^{2}$	A^{53}/T^{1}	A
	613	V^{212}/A^{3}	$T^{816}/I^{16}/A^{8}/V^{1}$	V^{54}	V
	627	E^{196}/K^{19}	$K^{838}/R^{2}/E^{1}$	K^{48}/E^{6}	K
	674	$A^{204}/S^{6}/T^{2}/G^{2}/E^{1}$	$T^{836}/A^{2}/I^{2}/P^{1}$	A^{54}	A
PB1	327	R^{147}/K^{3}	K^{766}/R^{66}	R^{58}	R
	336	V^{142}/I^{8}	I^{773}/V^{59}	V^{58}	V
PA	225	$S^{213}C^{1}$	$C^{829}S^{10}$	S	S
	268	L^{214}	$I^{827}K^{11}$	L	L
	356	$K^{212}X^{1}R^{1}$	$R^{827}K^{11}$	K	K
	382	$E^{208}D^{5}V^{1}$	$D^{824}E^{11}V^{2}N^{1}$	E	E
	404	A^{214}	$S^{828}A^{9}P^{1}$	A	A
	409	$S^{189}N^{24}I^{1}$	$N^{830}S^{7}I^{1}$	$S^{77}N^{1}$	S
NP	283	L^{372}/P^{1}	L^{7}/P^{643}	L^{61}	L
	293	R^{371}/K^{2}	R^{28}/K^{622}	R^{60}/K^{1}	R
	305	R^{369}/K^{4}	K^{636}/R^{14}	R^{61}	R
	313	$F^{371}/I^{1}/L^{1}$	Y^{642}/F^{8}	F^{61}	F
	357	$Q^{368}/K^{4}/T^{1}$	$K^{44}/R^{8}/Q^{1}$	Q^{61}	Q
	372	$E^{357}/D^{15}/K^{1}$	D^{630}/E^{23}	E^{61}	E
	422	R^{373}	K^{630}/R^{23}	R^{61}	R
	442	T^{372}/A^{1}	$A^{629}/T^{23}/R^{1}$	T^{61}	T
	455	D^{373}	$E^{630}/D^{22}/T^{1}$	D^{60}/E^{1}	D
M1	115	$V^{856}/I^{2}/L^{1}/G^{1}$	I^{981}/V^{9}	V^{88}	V
	121	$T^{840}/A^{19}/P^{1}$	A^{988}/T^{2}	T^{88}	T
	137	$T^{859}/A^{1}/P^{1}$	A^{974}/T^{12}	T^{88}	T
M2	11	$T^{434}/I^{11}/S^{2}$	I^{911}/T^{44}	T^{90}	T
	20	S^{471}/N^{13}	N^{926}/S^{29}	S^{90}	S
	57	$Y^{481}/C^{1}/H^{1}$	$H^{913}/Y^{33}/R^{2}/Q^{1}$	Y^{90}	Y
NS2	70	$S^{453}/G^{21}/D^{1}$	G^{903}/S^{2}	S^{61}	S

[1]Avian and human amino acid signatures in different viral genes of influenza A viruses as previously determined[20]. Numerical superscripts refer to the number of strains that possess those residues.

Table 3 Amino acid site residues associated with virulence in mammals in comparison with Egyptian isolates

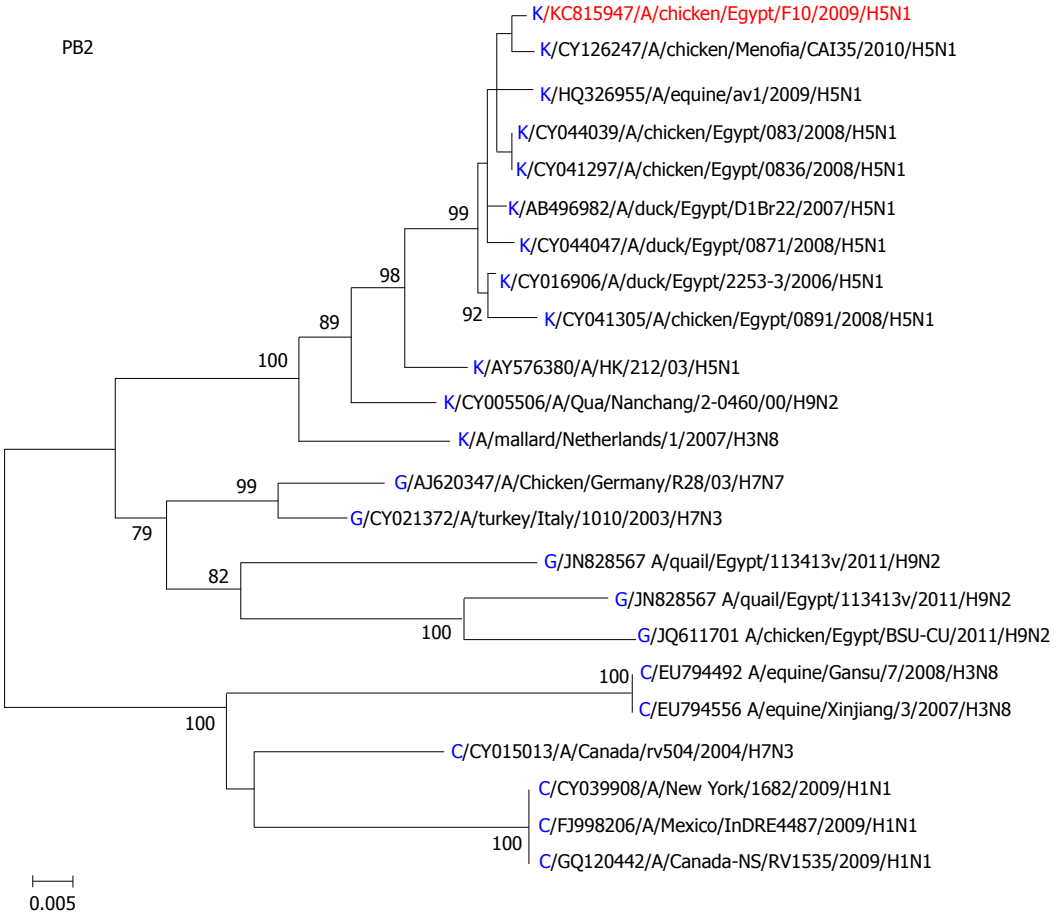
Gene	Site	Residue[1]		Egyptian	A/CK/	Ref.
		Virulent	Avirulent	H5N1 isolates	Eg/F10/09	
PB2	627	K	E	K	K	[11,27]
	701	N	D	D	D	[28]
PB1	317	I	M/V	M/V	M	[11,27]
PA	127	I	V	V	N.I.[2]	[25]
	336	M	L	L	L	[25]
M2	64	S/A/F	P	S	L	[17]
	69	P	L	P	P	[17]
NS1	42	S	A/P	S	S	[29]
	92	E	D	D	D	[27]
	97/92	E	D	E	E	[23]
	127	N	T/D/R/V/A	T/I	I	[30]
	189	N	D/G	D	D	[25]
	195	T/Y	S	S	S	[31]
NS2	31	I	M	M	M	[25]
	56	Y	H/L	H	H	[25]

[1]Virulent and non virulent amino acid residues refer to the ability of the virus to replicate in mammals as determined by Lycett *et al*[17]. [2]N.I.: Not included since it is not flanked by the primers used in the current study.

sequences of the seven genes of the A/chicken/Egypt/F10/2009 strain with other H5N1 strains from the Egyptian H5N1 isolates available in the flu database in order to screen amino acid signature and mutation trend change. Amino acid residues that have associated with mammalian virulence were also screened.

RESULTS

A/chicken/Egypt/F10/2009 in the current study is related to B2 sublineage. Eight amino acid substitutions were found in the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E. The different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains (Figure 1). The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are related to K, G, D, F, 1J, F and 1E genotypes respectively. Analysis of the *NA* gene revealed the presence of the 20-amino acid deletion (data not shown) and the presence of amino acid arginine (R) at position 110. The 228 (N to S) substitution is also present in the F10. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 showed avian like amino acid signatures (Table 2). The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains in comparison to the current escape mutant strain were screened and the residues were classified as virulent or nonvirulent (Table 3). Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues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89
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99
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79
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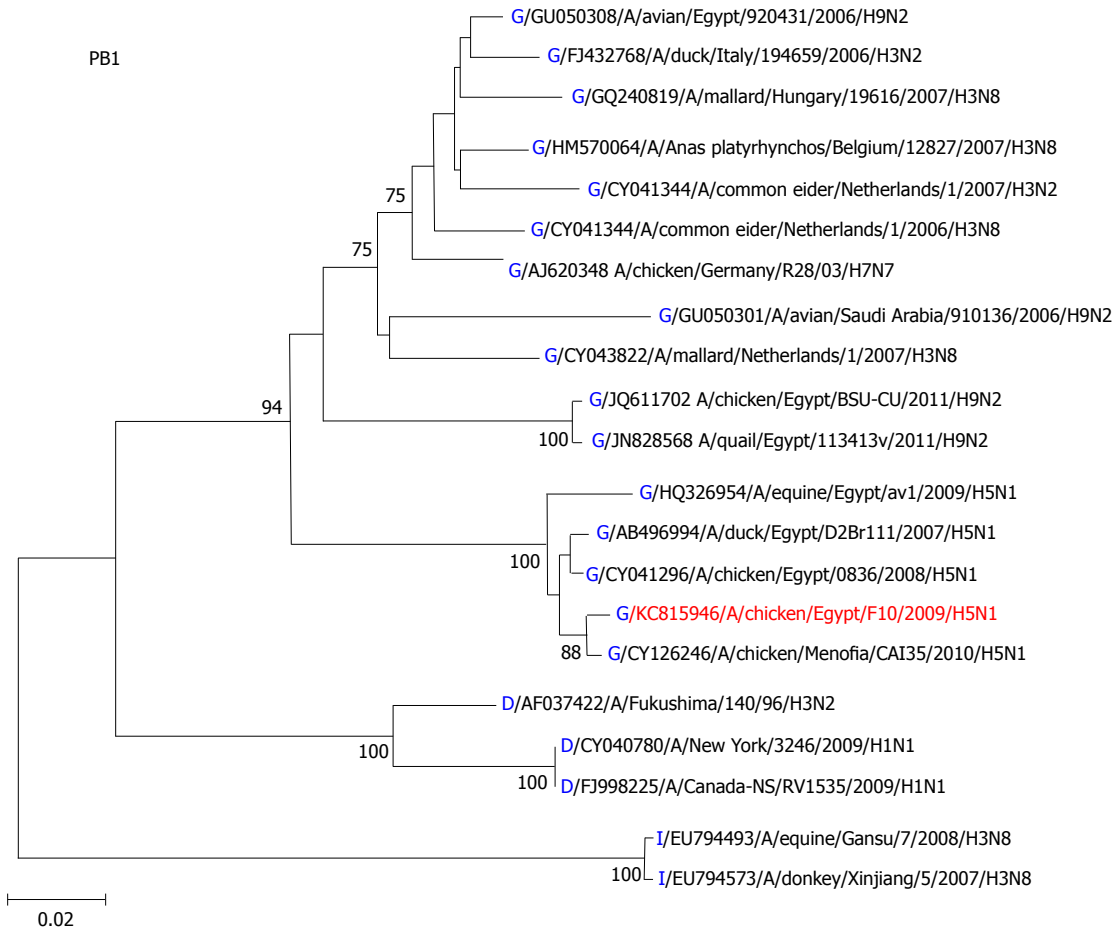
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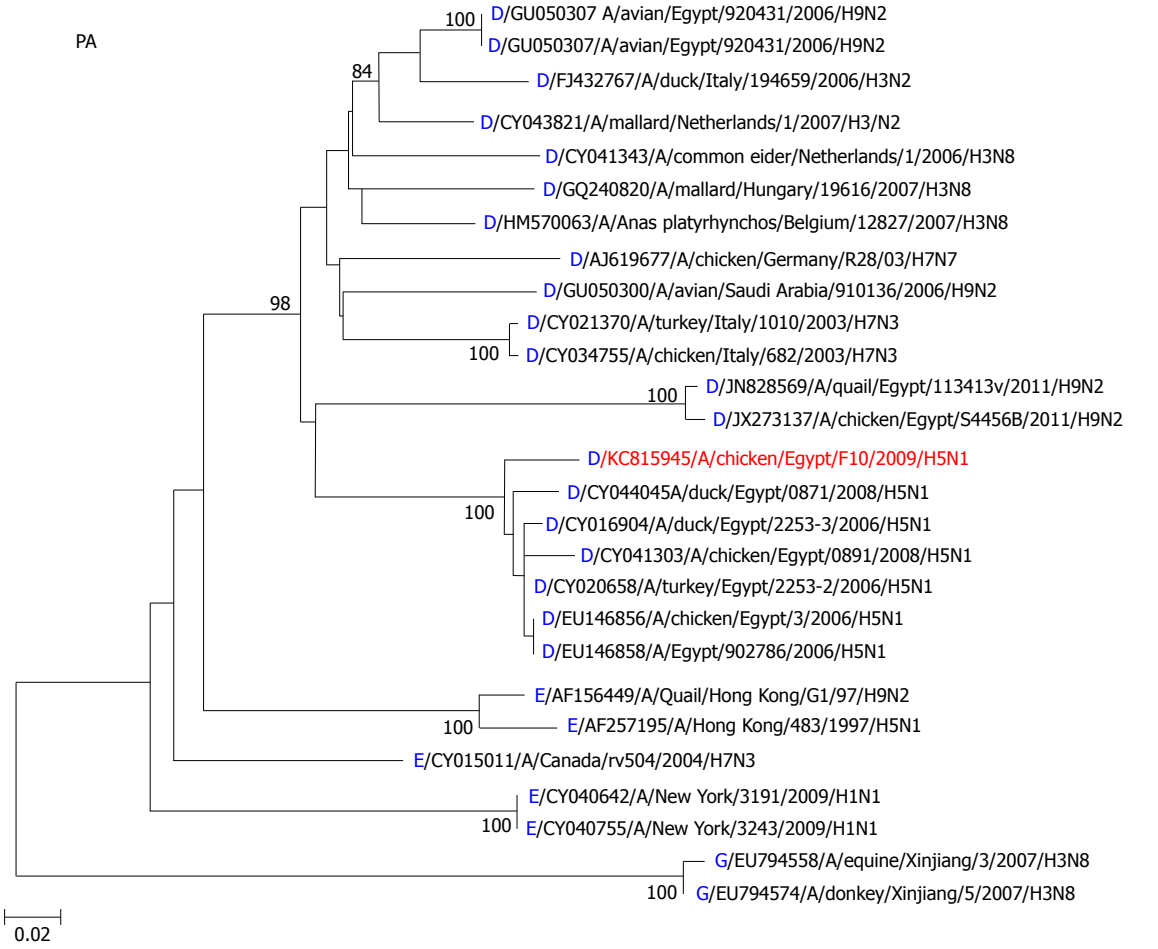
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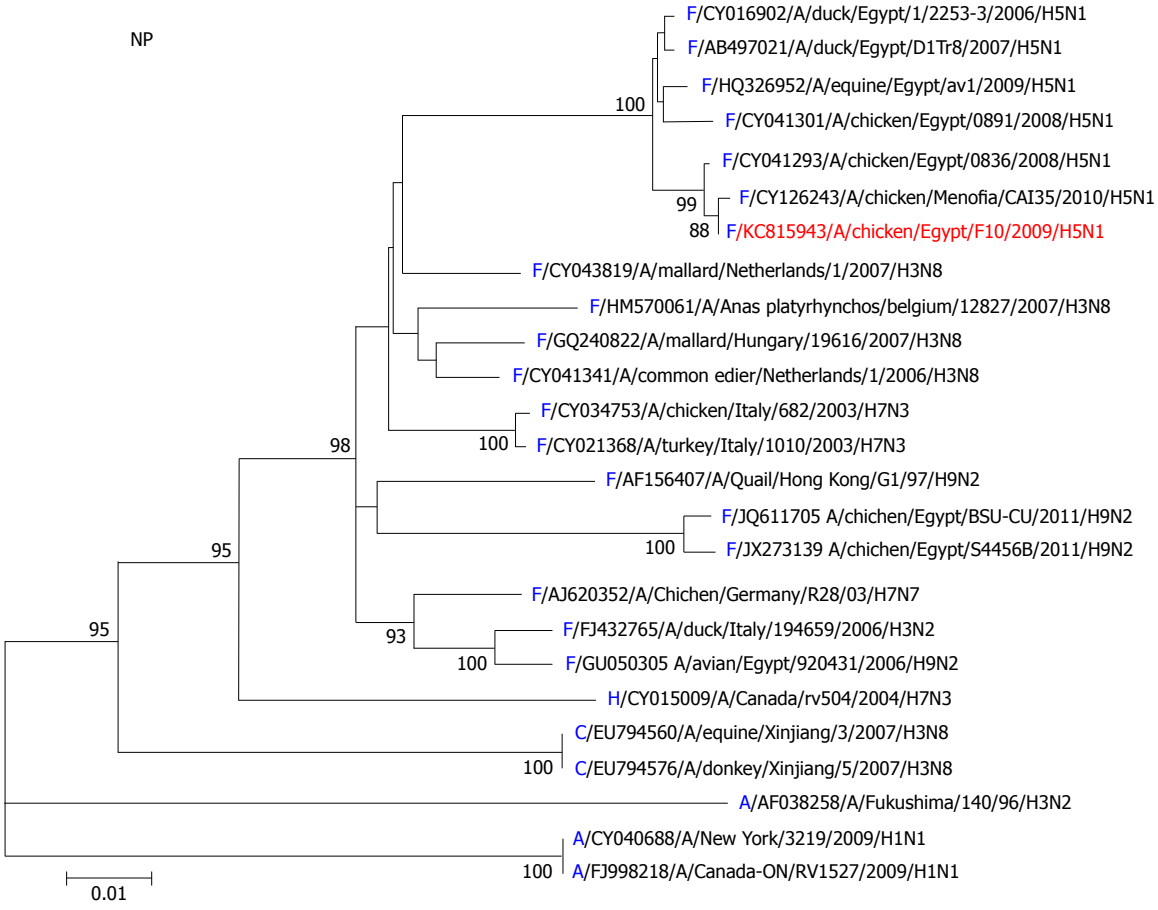
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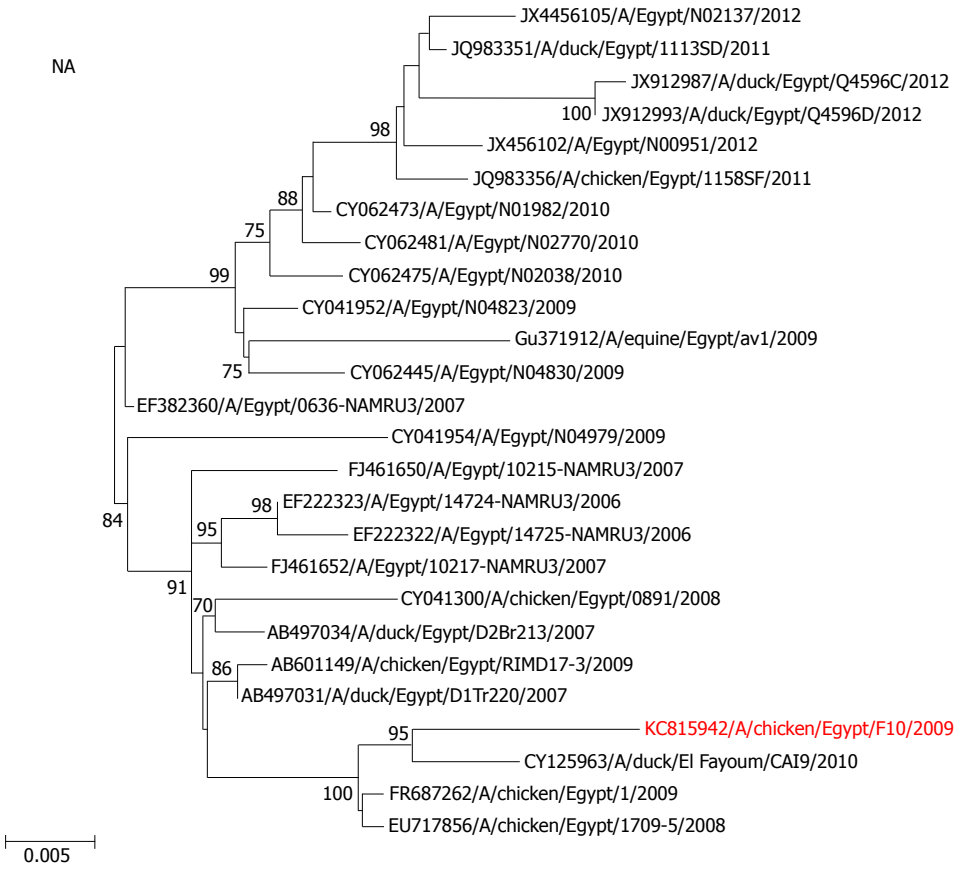
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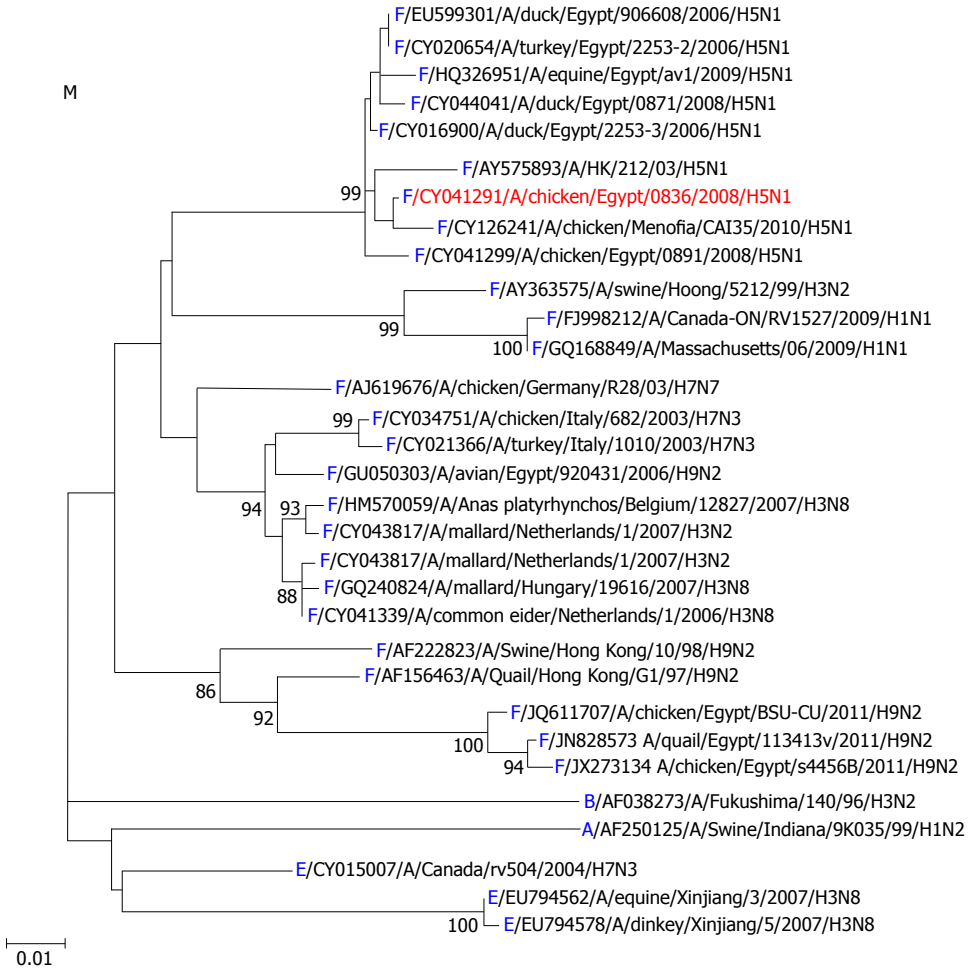
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F/CY020654/A/turkey/Egypt/2253-2/2006/H5N1
F/HQ326951/A/equine/Egypt/av1/2009/H5N1
F/CY044041/A/duck/Egypt/0871/2008/H5N1
F/CY016900/A/duck/Egypt/2253-3/2006/H5N1
F/AY575893/A/HK/212/03/H5N1
F/CY041291/A/chicken/Egypt/0836/2008/H5N1
F/CY126241/A/chicken/Menofia/CAI35/2010/H5N1
F/CY041299/A/chicken/Egypt/0891/2008/H5N1
F/AY363575/A/swine/Hoong/5212/99/H3N2
F/FJ998212/A/Canada-ON/RV1527/2009/H1N1
F/GQ168849/A/Massachusetts/06/2009/H1N1
F/AJ619676/A/chicken/Germany/R28/03/H7N7
F/CY034751/A/chicken/Italy/682/2003/H7N3
F/CY021366/A/turkey/Italy/1010/2003/H7N3
F/GU050303/A/avian/Egypt/920431/2006/H9N2
F/HM570059/A/Anas platyrhynchos/Belgium/12827/2007/H3N8
F/CY043817/A/mallard/Netherlands/1/2007/H3N2
F/CY043817/A/mallard/Netherlands/1/2007/H3N2
F/GQ240824/A/mallard/Hungary/19616/2007/H3N8
F/CY041339/A/common eider/Netherlands/1/2006/H3N8
F/AF222823/A/Swine/Hong Kong/10/98/H9N2
F/AF156463/A/Quail/Hong Kong/G1/97/H9N2
F/JQ611707/A/chicken/Egypt/BSU-CU/2011/H9N2
F/JN828573 A/quail/Egypt/113413v/2011/H9N2
F/JX273134 A/chicken/Egypt/s4456B/2011/H9N2
B/AF038273/A/Fukushima/140/96/H3N2
A/AF250125/A/Swine/Indiana/9K035/99/H1N2
E/CY015007/A/Canada/rv504/2004/H7N3
E/EU794562/A/equine/Xinjiang/3/2007/H3N8
E/EU794578/A/dinkey/Xinjiang/5/2007/H3N8
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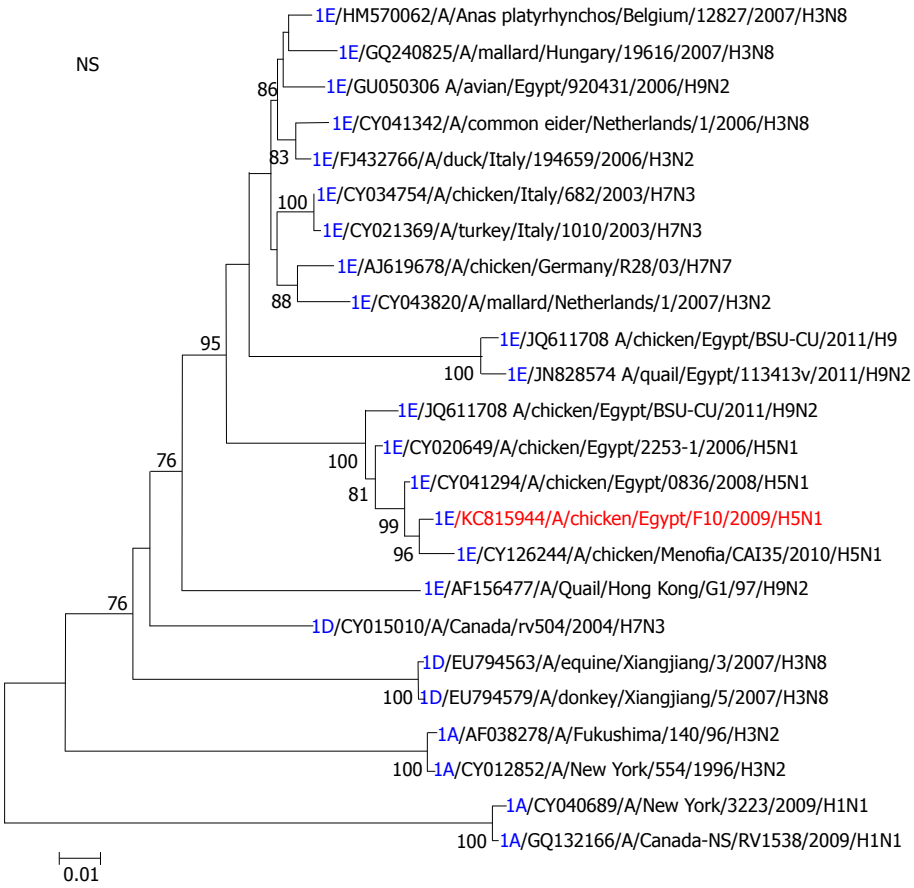

Figure 1 Phylogenetics of the viral genes of escape mutant H5N1 compared to selected influenza viruses. Escape mutant F10 strain examined in the current study was marked by a red color. The selected viruses were chosen to be representative from relevant sequences in GenBank database: H5N1 strains representative to the major gene lineages. Serotypes H1N1, H1N2, H3N2, H3N8, H7N3, H7N7 and H9N2 were also included in the phylogenetic trees of PB2, PB1, PA, NP, M and NS. For NA, Egyptian N1 sequences from Egyptian H5N1 strains were the only included sequences. The robustness of the individual nodes of the tree was assessed using a bootstrap of 1000 resembling in percent (70% and higher). Influenza A virus genotype tool (http://www.flugenome.org/ genotyping.php) was used to determine individual gene segment lineage. The genotype of each strain was mentioned in a blue color in the phylogenetic trees of PB2, PB1, PA, NP, M and NS.

in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion) was observed in this study in the F10 and also in other Egyptian H5N1 strains (Table 3). *PB2* of all Egyptian strains, including avian, mammalian isolates, possessed K627 (Table 3). F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in M2 protein while F10 possessed non virulent residue (L64) (Table 3). The *NS1* gene of F10 and other H5N1 Egyptian strains harbored L103F and I106M amino substitutions. The Egyptian H5N1 strains also possessed such amino acid substitutions (data not shown). Egyptian avian H5N1 strains including F10 possessed two human specific residues, E14 and R18 (data not shown).

DISCUSSION

Previous studies revealed that the *HA* genes from H5N1 Egyptian isolates were subjected to cumulative genetic drifts that resulted in further classification of the Egyptian strains into two sublineages [A(A1-A5) and B(B1-B5)][10]. A/chicken/Egypt/F10/2009 in the current study is related to the B2 sublineage. Eight amino acid substitutions were found in the Egyptian variants in lineage B, including the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E[10]. The deduced amino acid exchanges, as with most H5N1 Egyptian strains, showed polybasic cleavage motif consensus for clade 2.2 viruses, PGERRRKKR/GLF, while the consensus of 2.2, F10 PQGEGRRKKR/GLF, showed (R325G) substitution[10] with unknown significance. Lycett *et al*[17] specified 6 amino acid residues (86V, 124S, L/N138, T/S156, E/R212, T263) that are linked to the virulence of H5N1 in mammals. T156 and T263 were also present in F10 hemagglutinin[10].

In the current study, the different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains. The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are Eurasian in origin and related to K, G, D, F, 1J, F and 1E genotypes respectively[16].

Analysis of the *NA* gene revealed the presence of

the 20-amino acid deletion, a feature that is frequently observed during the process of adaptation of influenza viruses to poultry that are found to enhance the pathogenesis in chickens. The presence of amino acid arginine (R) at position 110 and the amino acid deletion in the *NA* are characteristic of clade 2.2 viruses[18]. The 228 (N to S) substitution is also present in the F10 and is an indication of 2.2.1 virus. Four *NA* mutations, E119G, H274Y, R292K and N294S, have been reported to confer resistance to *NA* inhibitors[19] but none were detected in the F10 isolate.

Chen *et al*[20] detected amino acid signatures specific to avian and human influenza A viruses. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 and most of Egyptian H5N1 strains showed avian like amino acid signatures.

Identification of the host range-specific amino acids could assume the functional sites that may mediate a host range. In a previous report, the amino acid sequences of the internal proteins in the Hong Kong poultry H5N1 viruses have been compared with those of other avian and human viruses[21]. The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains, in comparison to the current escape mutant strain, were screened and the residues were classified as virulent or nonvirulent; such residues have functional significance for virulence in H5N1 to mammals[17]. Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). An association between glutamic acid (E) at position 92 of the NS1 protein and resistance of H5N1 virus to interferons and TNF-α has been reported[22]. The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion)[23] was observed in this study in the F10 and also in other Egyptian H5N1 strains. However, Seo *et al*[22] 2004 reported that this substitution possesses low impact in the virulence in mammals. E627K substitution in the PB2 protein is one of the genetic indicators for the adaptation and efficient replication in humans[24,25]. The temperature sensitivity of the virus and the efficacy of viral replication depend on the amino acid residue 627 of PB2. Viruses showing K627 displayed higher activity of the polymerase complex during viral replication at a lower temperature in comparison to viruses displaying E627[26]. Efficient virus replication may explain the wide host range of subtype H5N1 strains and their high virulence[26]. The PB2 of all Egyptian strains, including avian and mammalian isolates, possessed K627.

We have compared the amino acid residues associated with H5N1 virulence in mammals[11,17,23,25,27-31] to their corresponding residues in the A/chicken/Egypt/F10/2009. F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in the M2 protein, while F10 possessed non virulent residue (L64). P42S and D97E amino acid substitutions in the NS1 are responsible for the virulence of H5N1 in mammalian species and cytokine resistance[22]. In addition, amino acid substitutions L103F and I106M were found to be adaptive genetic determinants for growth and virulence in the NS1 gene of both mammals and avian[32]; F10 and other H5N1 Egyptian strain harbored these amino substitutions. The G184 that was detected in F10 and other H5N1 Egyptian strains contributes to the cleavage and the polyadenylation specificity factor binding and strongly affected the viral virulence[33].

Amantadine resistance is associated with one of the following M2 residues: 26, 27, 30, 31, 34, or 38[34,35]; however, the Egyptian H5N1 strains did possess such amino acid substitutions. Human, swine and avian specific M2 residues were determined[36]. Egyptian avian H5N1 strains, including F10, possessed two human specific residues, E14 and R18[36,37].

The genetic characteristics of the H5N1 virus isolates from chicken in Egypt provided evidence of a high possibility of inter-species transmission. The examined escape mutant H5N1 strain carried no clear amino acid signatures from other Egyptian H5N1 strains.

COMMENTS

Background

Avian influenza viruses showed considerable capacity to cross species barriers to infect susceptible mammals, including humans. Point mutations and reassortment possess a crucial effect on the virulence of HPAI H5N1. Escape mutants resulting from antigenic drift of the viruses were selected under vaccination. The current study aimed to investigate whether the escape mutant strain (A/chicken/Egypt/F10/2009) possesses reassortant genes or amino acid signatures that differentiate it from other classical strains.

Research frontiers

The high error-prone replication of influenza viruses and vaccination pressure unequivocally enhance the robustness of mutation capacity of the influenza viruses. The amino acid signatures of the escape mutant strains have not been addressed. In this study, the authors demonstrate the genetic constitution of the escape mutant strain and the possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

Innovations and breakthroughs

Recent reports have highlighted critical amino acid substitutions in different alleles of influenza viruses that are associated with virulence to mammals. Amino acid signatures specific to avian and human influenza A viruses were also determined in previous reports. This study reported the presence of different amino acids substitutions in different alleles related to virulence to mammals; however, it failed to find the presence of prominent amino acid signatures in the examined escape mutant strain.

Applications

By understanding the amino acid substitutions in H5N1 escape mutants, its impact on virulence to mammals and how it could be accelerated under vaccination pressure, the avian influenza control procedure method based on vaccination should be reevaluated.

Terminology

Mutation at the *HA* epitope region is among the strategies the influenza virus uses to escape the immune system and represents the most important hindrance to vaccine development. Meanwhile, mutations in the other viral alleles play a crucial role in modulating virus pathogenicity to the original hosts and inter-species transmission to mammalian species, including humans.

Peer review

The authors studied the genetic constitution of the escape mutant H5N1 strain

in comparison with other influenza viral strains. Possible amino acid signatures were explored for identification of the escape mutant from other Egyptian H5N1 and different proteins with amino acid substitutions were also recorded compared to the available Egyptian H5N1 strains. The paper's scientific content is original and of good quality as a research article.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Conference paper

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases.

Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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