Viruses and virus-like particles identified in ostrich gut contents

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ABSTRACT

Samples of either gut content from ostriches showing symptoms of enteritis, or allantoic fluid of eggs inoculated with ostrich isolates, were examined for the presence of viral agents by direct negative-contrast electron microscopy. Only a few virus types could be identified with certainty, namely a circovirus (1 sample), a coronavirus (1 sample), a member of either the toga- or bunyaviridae (1 sample), enterovirus (16 samples) and paramyxovirus (26 samples). A large number of samples contained structures resembling myxovirus particles that were interpreted as fringed membranous particles of non-viral origin. An unusual observation of probable single-strand nucleocapsid helices, possibly originating from digested plant material and which were identified in a number of small intestine samples, is reported. This is the 1st report of a spectrum of viruses and virus-like particles occurring in enteric samples from ostriches in South Africa. The low incidence and variety of viruses reported here contribute to the multifactorial origin and complexity of enteric disease in ostriches as well as in other birds and mammalian species.

Key words: enteritis, fringed membranous particles, ostrich, paramyxovirus.

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INTRODUCTION

The aetiology of diarrhoea/enteritis outbreaks among birds is not always established: illness is usually attributed to combinations of management errors, nutritional deficiencies and imbalances or infectious agents including viruses, bacteria and/or protozoa. Research on infectious agents affecting ostriches, which often cause high mortalities, has gained momentum in recent years with the expansion of the ostrich farming industry. Maintenance of healthy stock has become very important and prompted studies of possible causative factors involved in the enteritis complex, including virus infections. This paper describes viruses and virus-like particles observed in the gut content of ostriches (obtained from live sick birds or at necropsy performed on birds with enteritis symptoms) and allantoic fluid (from eggs after inoculation with ostrich isolates) presented for the diagnosis of infectious agents by electron microscopy. In many samples the presence of varying amounts of virus-like particles made a precise diagnosis difficult. In addition, apparent single-strand nucleoprotein helices of possibly plant viral origin were encountered, which further complicated accurate diagnosis.

MATERIALS AND METHODS

Samples were collected from sick birds and necropsies originating from farms throughout the Little Karoo during the period October 1993 to October 1997. These samples were dispatched from the veterinary laboratory of the Klein Karoo Koöperasie in Oudtshoorn *via* an overnight courier service. After low speed centrifugation (3000 g, 20–30 min) of 15 mℓ samples (diluted when necessary) to remove debris and bacterial components, the supernatant was centrifuged at 20 000 g for 1 h. Since 1997 a microlitre centrifuge utilising 1.5 mℓ Eppendorf tubes has been used with effective g-values of 600 and 15 000, reducing the previous times to 15 min and 30 min respectively[4]. The resulting pellet (using either technique) was rinsed in distilled water, suspended in a water droplet and negatively stained with 3 % phosphotungstic acid (PTA), pH 6.4, on formvar-carbon coated grids following standard procedures. An insignificant background of (cellular) debris of unknown origin (digested material?) and varying amounts of bacteriophages and bacterial remnants were regularly observed.

RESULTS AND DISCUSSION

From October 1993 to October 1997 a total of 348 samples were examined, divided into 2 groups as shown in Table 1. Of these, 104 samples were obtained from either tissue-culture fluid, allantoic fluid or amniotic fluid after inoculation with organ fractions or gut content from ostriches. The other 244 samples represented gut content (mainly the small intestine) obtained from sick birds or *post mortem* cases of which the ages were not always available. In 105 of the 348 samples no specific types of (virus) particles were identified. In the remaining samples only a few virus types could be positively identified, namely a circovirus (Fig. 1a), 17–22 nm in diameter (1); coronavirus (Fig. 1b), 120–160 nm in diameter (1); a spherical virus (Fig. 1c) 70–90 nm in diameter with morphology resembling either the toga- (<70 nm) or bunyaviridae (80–120 nm) and at present subject to further investigation to determine its proper classification; enterovirus (Fig. 1d), 30 nm in diameter (16) and paramyxovirus particles (Figs 1e, 2) discussed below.

Paramyxovirus particles

Twenty-six samples of allantoic fluid were identified as positive for paramyxovirus (PMV). The particles observed in these samples were >150 nm in diameter, usually spherical but also pleomorphic or filamentous and enveloped (Table 1, Group A). In addition, these samples revealed typical 13–18 nm 'herring-bone' nucleoprotein strands (= nucleocapsid) (Fig. 1) which are considered to be diagnostic for paramyxovirus (PMV). At least 2 of the above cases were confirmed clinically to be associated with localised outbreaks of Newcastle disease among ostriches. Some PMV particles displayed bizarre shapes, with long filamentous extensions being observed, while others differed markedly in size (Fig. 2). Much of the pleomorphism observed by negative staining appears to be induced during storage or during preparation of the samples for electron microscopy[12]. This phenomenon has also been observed in our laboratory during the routine preparation of virus samples.

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Table 1: **Distribution of virus particles in ostrich samples.**

A: Tissue culture fluid/allantoic fluid/amniotic fluid.

Year	Number of samples	Samples not diagnosed	Corona-virus	Paramyxovirus (PMV) + 'herring-bone' strands
1993	0	—	—	—
1994	10	4	—	6
1995	36	27	—	9
1996	52	44	1	7
1997	6	2	—	4
Total	104	77	1	26

B: Small intestine contents.

Year	Number of samples	Samples not diagnosed	Chicken anemia virus	Toga/Bunya-virus	Entero-virus	[a]FMP + + zigzag strands	[a]FMP/PMV + + zigzag strands
1993	24	—	—	—	3	20 + 6[b]	1 + 10
1994	88	8	—	—	5	46 + 14 only z: 4	28 + 7
1995	96	12	1	1	5	54 + 15 only z: 2	27 + 6
1996	34	8	—	—	3	18 + 5	8 + 0
1997	2	—	—	—	—	2 + 1	—
Total	244	28	1	1	16	140 z = 47	64 z = 13

[a]No 'herring-bone' strands were observed in the FMP, FMP/PMV samples.
[b]20 + 6 etc. means 20 specimens showing FMP particles of which 6 show zigzag strands as well.

Fringed membranous particles (FMP) and fringed membranous particles/ paramyxovirus-like particles (FMP/PMV)

A large number of samples contained particles designated FMP (fringed membranous particles: Figs 3–5; Table 1: Group B – 140 samples) or FMP/PMV (fringed membranous particles/paramyxovirus-like particles: Fig. 6; Table 1: Group B – 64 samples). The samples showing FMP revealed masses of pleomorphic, fringed membranous particles that varied noticeably in appearance, shape and size (100–500 nm) but resembled negatively-stained myxovirus particles.

The 64 samples showing FMP/PMV similarly revealed particles in which a definitive diagnosis could not be made. Apart from some spherical or pleomorphic forms typical of the fringed membrane particles described above, many particles (Fig. 6) had a clear fringed filamentous form that resembled known paramyxoviruses, *e.g.* Newcastle disease virus (NDV) isolated from chicken-egg allantoic fluid (Fig. 7).

The negatively-stained particles in these 2 categories thus presented difficulties with regard to their correct identification. They could be interpreted either as possible FMP or PMV, but doubt as to their viral origin was expressed. For instance, although apparently disrupted particles were frequently observed, no firm evidence of any internal component was obtained. Since PMV particles are similar in appearance to FMP and not easily distinguished from them, they are likely to be overlooked against a background of FMP. Further distinction based on the fringe diameter of PMV and FMP is difficult, since the various fringe size ranges overlap: the diameter of the fringe measures 10–18 nm in paramyxovirus, 12 nm in orthomyxovirus and 12–18 nm in the FMPs.

In addition, no 'herring-bone' strands diagnostic for a paramyxovirus were observed in any of these samples (Table 1: FMP 140; FMP/PMV 64). It is not possible to positively identify paramyxoviruses, unless the 17 nm helical nucleocapsid ('herring-bone' strands) are observed. The situation is complicated by the fact that single-strand helices (orthomyxovirus) or herring-bone strands (paramyxovirus) are not necessarily observed in the presence of positively known myxovirus particles (personal observations). This phenomenon has obvious implications with regard to the interpretation or identification of the type of fringed particle present in some samples. Pleomorphic, fringed particles[6,7,13] (Figs 3–5) can easily be distinguished from coronavirus[13] (120–160 nm, Fig. 1b) or togavirus (<70 nm, Fig. 1c) particles. However, without the release of visible nucleocapsids from paramyxoviruses or orthomyxoviruses, it is difficult to distinguish them from FMPs.

The distinction and identification of PMV from FMP is further complicated by some experimental factors. The variability noticed in the appearance of the FMP particles (Figs 3–5; Table 1: FMP 140; FMP/PMV 64, Fig.6) is probably due to numerous factors affecting the staining result, such as the type of material analysed, concentration of particles in the sample, phase of infection, processing of the sample and properties of the stain. For example, the pH of the aliquot and ionic condition of the sample affects staining. The time factor is also important: denaturation may occur from collection onwards, altering the structure of the particles. Some particles (including the fringe) already appeared to be lysed to various degrees (Figs 3, 4). The negative stain (PTA) can be detrimental to viral particle structure: the differential loss of subunits resulting from the stain used or lysis causes variations in the length and composition of the fringe (fringe part of membranous structure) of the FMPs. In

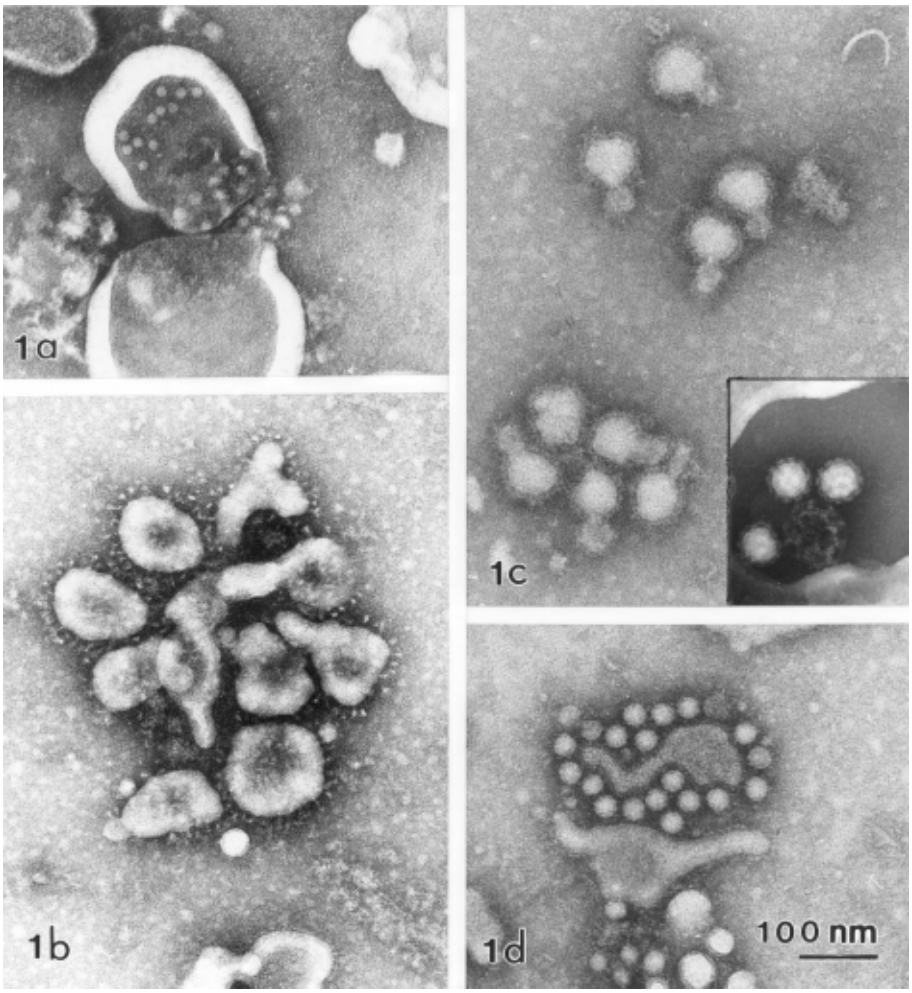

Fig. 1a: **A group of circovirus-like particles, spherical/ icosahedral in structure; surface detail is not apparent.**
Fig. 1b: **Typical grouping of coronavirus particles, spherical/ pleomorphic in shape, enveloped with distinctive club-shaped surface projections.**
Fig. 1c: **Spherical virus particles with either toga/bunyavirus morphology. Inset: the appearance of the toga/bunyavirus particles when fixed in glutaraldehyde and then negatively stained as before. The particles reduced in size from 70–90 nm to 62.5 nm spherical particles with morphology resembling Semliki forest virus, suggesting that the particles in this micrograph belong to the togaviridae.**
Fig. 1d: **Typical appearance of enterovirus particles, icosahedral in structure, with negative staining.**

The bar in Fig. 1d represents the same scale for all virus-like particles in Figs 1a–d.

some instances the fringe appeared to be more compact, with closely-spaced petal-shaped or rectangular surface projections often being observed. Any combination of the above factors resulted in an inconsistent appearance of fringes between particles in the same field.

These observations posed some unresolved questions with regard to the interpretation of fringed particles as of viral or non-viral origin and prevented a precise viral diagnosis. A positive identification of either ortho- or paramyxovirus must be viewed with extreme caution unless distinguishing features and criteria can be established or discerned for the unequivocal distinction between myxoviruses and FMPs. Therefore, in the absence of 'herring-bone' strands typical of PMV particles, the fringed particles observed in these samples (Table 1, Group B: 140 FMP; 64 FMP/PMV) are at present diagnosed as representing FMPs.

Nature/origin of FMP

An association between the presence of FMPs and enteric disease has yet to be clearly established. These particles have been described as 'duodenal brush border vesicles' (in comparison with coronavirus[13]), or as fringed membranous particles (FMP) (virus infections in turkey poults[6]), 'fimbriated' virus-like particles (virus particles in game birds[7]) or as membrane ghosts and glycocalyceal bodies[9]. We often observe FMPs in faeces samples from dogs, cats and cattle but rarely in horses. The current opinion is that the fringed particles could originate from fragmentation of the epithelial cells lining the intestine, related to abnormalities in intestinal structure and function[13]. This suggestion about their origin may also explain the generally large number, size variation and pleomorphism of the observed fringed particles.

Z or zigzag strands or single-strand helices

An additional type of structure was observed in a number of samples. In 47 of the 140 FMP samples and in 13 of the 64 FMP/PMV samples (Table 1, Group B), zigzag-like strands, or more correctly, single-strand helices, in varying quantities and of variable lengths, even in the total absence of cellular debris or

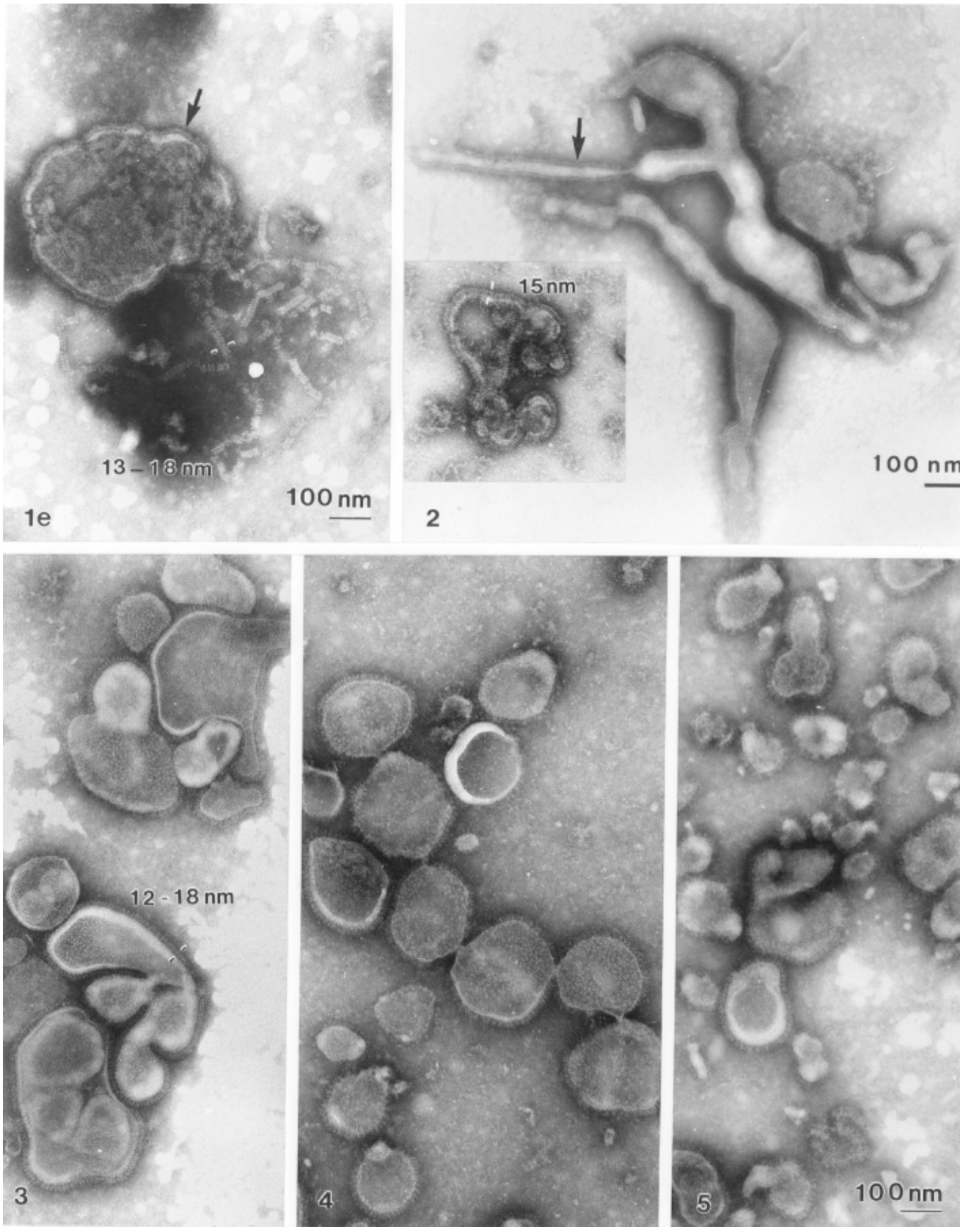

Fig. 1e: **Typical paramyxovirus particle showing the release of 'herring-bone' nucleoprotein strands.**
Fig. 2 (and inset): **Pleomorphic paramyxovirus particles. Note the variation in the appearance of the fringe** (arrows) **in comparison to that in Fig. 1e.**
Figs 3–5: **Fringed membranous particles** (FMP). **Note the variation in shape and appearance of the fringe of the different particles.**

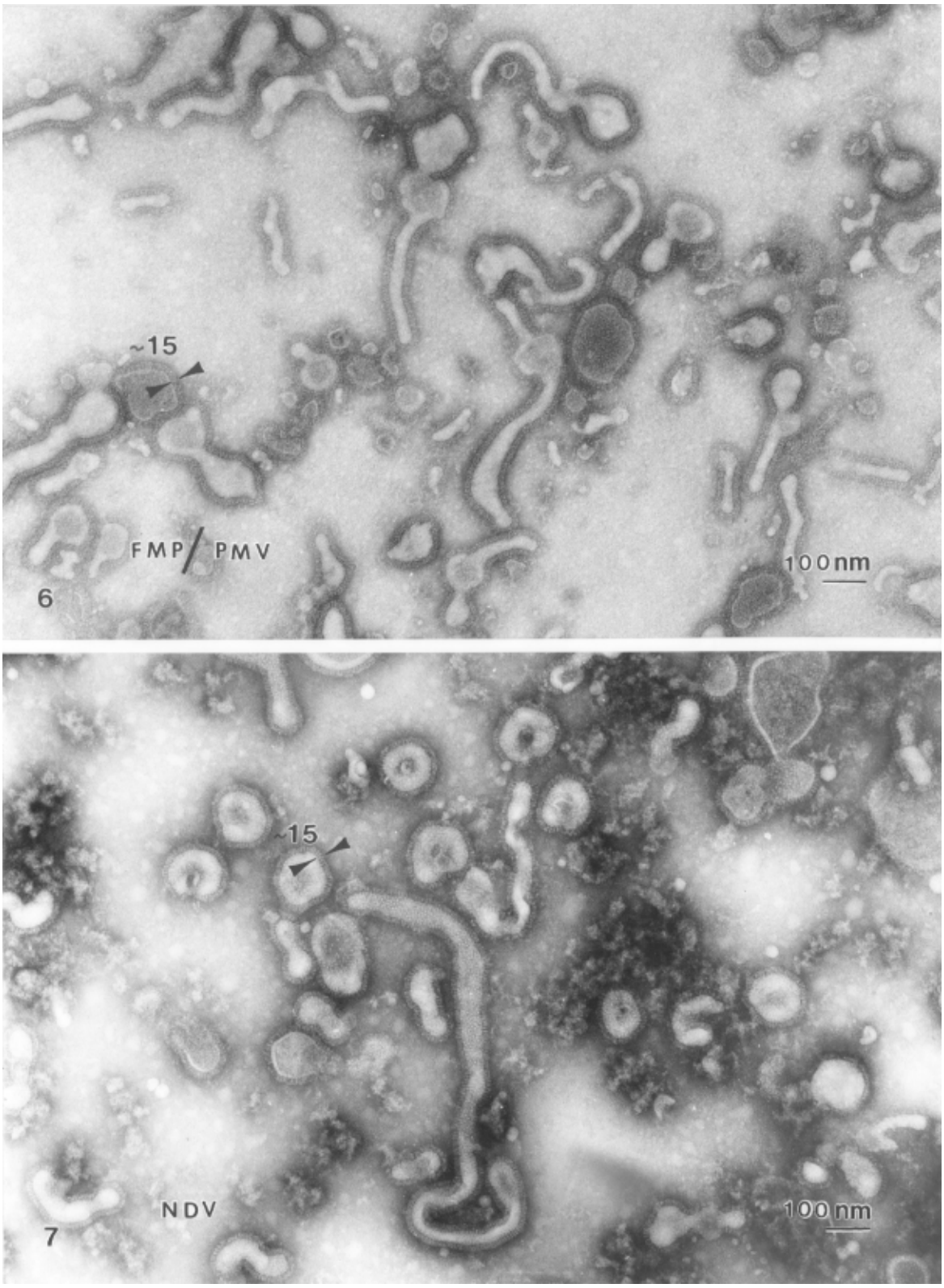

Fig. 6: **Filamentous FMP/PMV. Compare the appearance of the fringe to that illustrated in Figs 3–5.**
Fig. 7: **Newcastle disease virus** (NDV) **particles isolated from allantoic fluid.**

bacteriophages, were observed (Figs 8–11). They were termed z or zigzag strands to distinguish them from myxovirus helices[10]. The strands took on a number of forms. The most common pattern observed was a single strand, 15–17 nm in diameter, in the form of an expanded helix (Fig. 8); the next most frequent arrangement was a criss-cross pattern interpreted as 2 of the strands entwined in a double helix (Fig. 9). In Fig. 10, 2 single strands can be seen emerging from a double helix segment while in Fig. 11 a compound helix is shown, interpreted as 2 double helices entwined to form a higher order helix. Remnants of a particle were rarely observed associated with strands (Figs 9, 10, 11: inset). No evidence

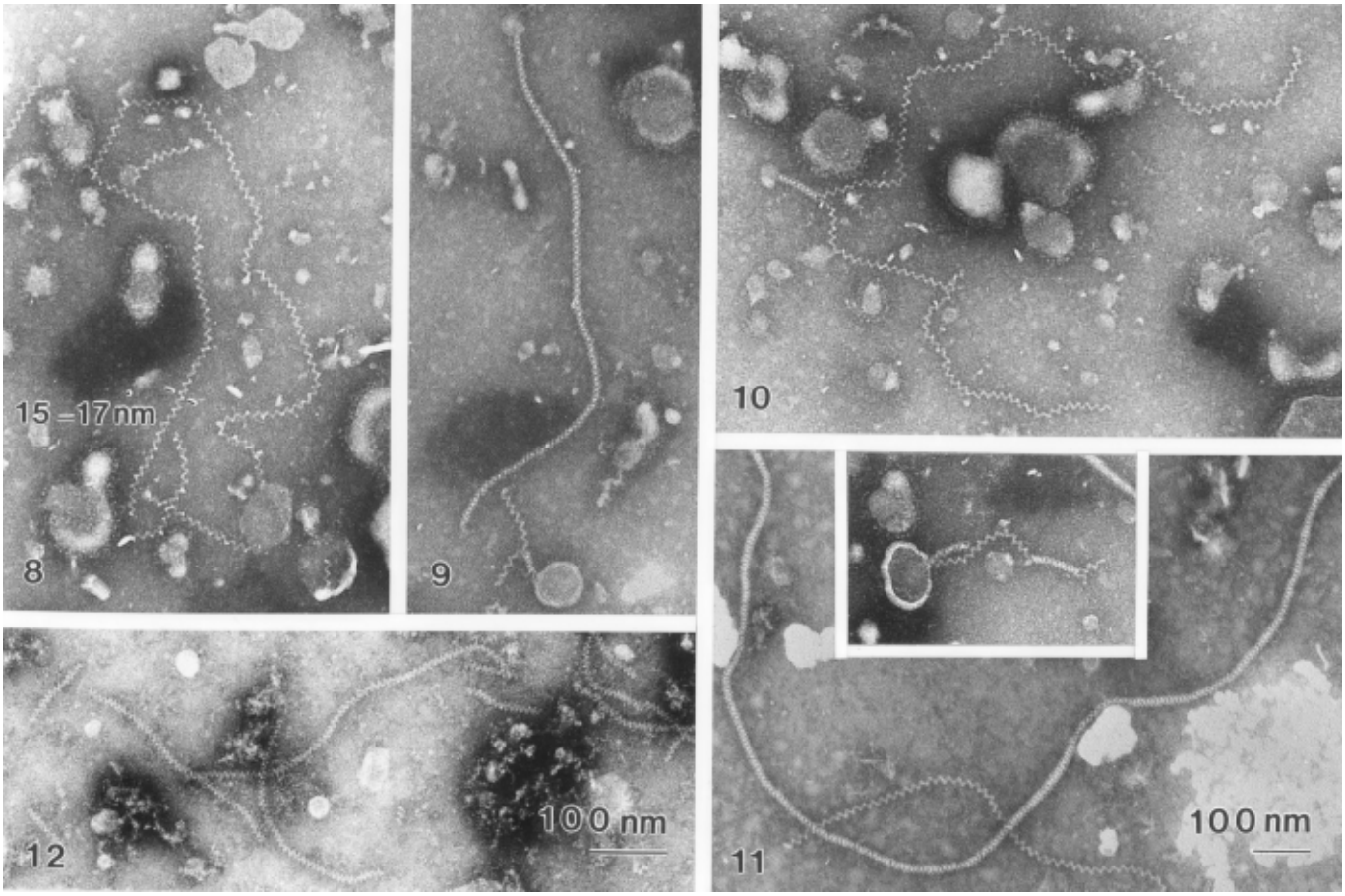

Fig. 8: **Single-strand helices** (termed z- or zigzag strands to distinguish them from myxovirus helices[10]).
Fig. 9: **Double coil helix** (from z-strands) **with remnant of particle from which it may have originated.**
Fig. 10: **Two single z-strands emerging from a double-coiled segment.**
Fig. 11: **A compound** (z-) **helix formed by 2 double-helix strands. Inset: note double-coiled segment and its association with a particle.**
Fig. 12: **The z-strands from a horse, although of different pitch and length, showing similarities to those observed in the ostrich material.**

The z strands in Figs 8–11 are from the 47 FMP samples and the 13 FMP/PMV samples (Table 1, group B).

of release from a particular type of particle, bacteria or bacteriophages, was observed.

Nature and origin of zigzag strands (single-strand helices)

At present we are of the opinion that these clearly-defined strands resemble typical nucleocapsid helices associated with, as yet unknown, viral particles. The features and appearance of these strands when compared with protein filaments (*e.g.* actin) suggests that they conform more to nucleoprotein strands (see discussion below). There are a number of possibilities regarding the origin of the single helical strands:

A. One could envisage that observation of zigzag strands in allantoic fluids would be conclusive for their animal viral origin. However, preliminary inoculation of eggs with a few gut samples (from the FMP/PMV group) revealed no viral growth or the presence of zigzag strands after 3 passages. A conclusive answer would require a more complete approach following all routes of inoculation.

B. The presence of an orthomyxovirus (OMV). Avian influenzavirus (AIV), for instance, is known to infect the respiratory organs. The unlikely presence of this virus in gut fluid may be due to the swallowing of mucus from the respiratory tract, but this would not account for the numbers of fringed particles or the masses of z strands observed in the FMP and FMP/PMV samples. The helical nucleocapsid of influenza virus, composed of 8-segmented helices measuring 50–100 nm in length and 7–10 nm in diameter, is only released as a single-strand helix using special methods[10]. These helices are zigzag strands of different length and pitch from the strands reported in this study and completely different from paramyxovirus 'herring-bone' strands. This supports our interpretation of a zigag strand as a nucleocapsid helix. In addition, the nucleocapsid of influenza virus is extremely fragile. This implies that it will not survive in ostrich gut contents nor withstand normal preparation procedures. Therefore an orthomyxovirus can be excluded as the origin of zigzag strands.

C. Assembly of unknown identical structural (nucleo)protein units present in the gut fluid of ostriches. It could be argued that zigzag strands could result from the assembly of excess fringe structures appearing on FMP or myxoviruses or the assembly of identical structural units known to occur on bacteriophages, flagella or bacterial cell walls[8]. However, the assembly of fringe or other viral protein units does not form defined helices. Moreover, the type of strands or assembly complexes released from bacteria or bacteriophages are known and do not resemble zigzag strands[8], and would be expected to have a more regular appearance both in ostrich gut contents and in faeces samples of infected mammalian species.

D. A 4th hypothesis may be the most

convincing and plausible. Similar z strands of a different pitch and length (Fig. 12) were observed in a sample obtained from a horse. This observation led to the hypothesis that these particular z strands may originate from digested plant material infected with rod-shaped viruses. Morphological support for this hypothesis can be obtained from studies on plant viruses[2,3], where the authors observed, in sectioned material, long, clearly-defined expanded helices that were thought to be components of the virus rods in the process of assembly[2,3]. The single helices are of similar diameter (*c.* 15 nm) and length to those reported in the present study. Some plant viruses can occur in quantities of 10^6/cell, explaining the numbers of strands observed.

CONCLUSIONS

There is in our opinion enough evidence for the interpretation of the zig-zag strands from ostriches (and the horse) as viral nucleoprotein strands released from digested plant material. It seems unlikely that the strands originate from the epithelial lining of the hosts' digestive tract. It is therefore proposed that the FMPs are of host epithelial origin and the z strands of plant virus origin.

Finally, this survey of possible viral agents associated with enteritis in ostriches has revealed:

1. Samples in which no viral identification could be made, indicating that there are other factors or infectious agents present and responsible for the enteric conditions in these ostriches.
2. The diagnosis of relatively easily-identified virus particles *e.g.* coronavirus particles (low incidence) could easily be masked by FMPs. This suggests that there are probably other viruses that remain to be identified in ostriches[1].
3. A large group of samples diagnosed as containing non-viral FMPs, which prevented a precise viral diagnosis being made and which probably complicated the identification of PMV particles. However, we are confident that the correct diagnosis has been made in describing these particles as FMPs.
4. An unusual presence of single-strand nucleocapsid helices of unconfirmed viral origin external to the host.

This work and results from other studies[1,5,11] suggest that outbreaks of enteric disease among young turkeys[6], chickens and ostriches (as well as various mammalian species) are not always attributable to a solitary agent. Typical examples that would illustrate the multifactorial origin of the enteric disease complex are represented by infectious agents such as *E. coli* and rotavirus in weaner piglets[5] and several viruses considered responsible for the 'runting and stunting' syndrome of chickens[11]. Furthermore, the same agent is not necessarily always present or detectable each time birds develop gastrointestinal disease, or each time their gastrointestinal contents are examined by electron microscopy.

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